# AN IMMUNOCHEMICAL ANALYSIS OF FACTORS AFFECTING FERTILITY

M.H. Johnson

Christs' College, Cambridge

1969

List of Contents


| | | Page |
|---|---|---|
| List of Contents | | i |
| Preface | | ii |
| Acknowledgements | | iii |
| Summary | | v |
| Introduction | | 1 |
| Chapter 1. | A naturally-occurring antibody to spermatozoa. | 26 |
| Chapter 2. | An immunological barrier in the testis. | 42 |
| Chapter 3. | Evidence that the immunological barrier in the testis affects the extent and pattern of development of autoimmune aspermatogenesis in the guinea-pig. | 53 |
| Chapter 4. | Detection of a change in the permeability of the immunological barrier of the guinea-pig testis following isoimmunization with testis. | 72 |
| Chapter 5. | The effect of weakening the testis barrier or of increasing the immune response on the induction of autoimmune aspermatogenesis. | 89 |
| Chapter 6. | An immunological barrier in the adnexal glands of the male genital tract of animals and man. | 102 |
| Chapter 7. | The effect of immunological barriers in the female genital tract on the depression of fertility by systemic antibodies to spermatozoa. | 119 |
| Conclusion | | 134 |
| References | | 140 |

# PREFACE

The dissertation is presented in two volumes, this volume containing the text and the second volume containing figures and tables arranged in their order of reference. The legends to figures are on the left-hand sheet of the open volume and face the figures that they describe. On the reverse side of the same sheet in the top right-hand corner is the figure code (e.g. F19/20 for figures 19 and 20) for rapid location of figures. The tables are interspersed amongst the figures with a table code (e.g. T1 for table 1) in the top right-hand corner. A brief index at the front of volume 2 indicates the position of the tables relative to the figures.

The surgical operations for the cannulation of the ram testes were done jointly with Dr. B.P. Setchell at the Institute of Animal Physiology, Babraham; the work with the uterine diffusion chambers was done with Dr. R.G. Edwards, Dr.L.E. Rowson, Dr.D. Israelstam, Dr. D.V. Nino and Dr.L. Talbert.

## ACKNOWLEDGEMENTS

My grateful thanks are due to Dr. R.G. Edwards for excellent advice and supervision, and for the interest and stimulation of our many discussions. My thanks also to Brian Setchell and Professor C.R. Austin for their full and helpful discussions, and to Professor Austin, Professor A.S. Parkes and Professor Sir Bryan Matthews for allowing me to work in the Marshall Laboratory.

I am indebted to Sally Fawcitt for technical assistance, to Valerie Hunn for help with photography, to John Lee and Beryl Smith for looking after animals, to Margaret Deamer and Pat Rubbra for typing the thesis, and to all other members of the Marshall Laboratory for their patience.

I am grateful to Dr. P.J. Lachmann, Dr. A. Munro, Dr. R. Skaer, Dr. R. Clarke and Dr. M. Hobart for the generous loan of material or equipment.

The work was financed by a Medical Research Council Research Scholarship, a Research Fellowship at Christ's College, Cambridge,

and by grants from the Medical Research Council to Professor C.R. Austin and from the Ford Foundation to Professor A.S. Parkes and Professor C.R. Austin.

## SUMMARY OF DISSERTATION

The injection of homologous testis into mature males and females of mammalian species induces an immune response to spermatozoa. This dissertation suggests that a major determinant of the degree and type of resultant damage is the presence of barriers isolating the contents of the genital tracts immunologically.

Normal sera of many species contain a low-titre antispermatozoal activity which is identified as an antibody. This antibody is prevented from entering the male genital tract by an immunological barrier which is highly effective in the testis, less so in the rete testis and ductuli efferentes and weakest in the accessory glands. The effect of the testicular barrier may be overcome by boosting the antibody level and by weakening the barrier of cadmium, trauma or local inflammation. The differential effectiveness of the barrier results in two types of immunologically induced male infertility. Spermatogenesis may be unimpaired, but antibody is transmitted into the seminal plasma and agglutinates ejaculated spermatozoa. A fiercer immune response to spermatozoa provokes an inflammation at the rete testis. The inflammation reduces the efficiency of the adjacent seminiferous tubule barrier as shown by following changes in the intratesticular distribution of the dye acriflavine. The use of acriflavine is shown to be a legitimate method for evaluating barrier changes

by studies on hypophysectomised and cadmium treated animals. The leakiness of the seminiferous tubules following inflammation permits readier entry of the specific immune response to spermatozoa, which reacts with intratubular antigen. More inflammation results and the damage spreads autocatalytically through the testis.

Little serum protein is present in the female genital tract, but levels are dramatically increased following the insertion of a chamber for collection of uterine fluid and may resemble those in the presence of an IUD. Follicular fluid contains most of the serum proteins and some of these, including immunoglobulins, are retained in the sponge of follicular cells surrounding the ovulated egg. It is suggested that in the female the maximum antifertility effect of antibodies to spermatozoa will be exerted at the point of fertilization.

# INTRODUCTION

The complexity of the growth, differentiation and maintenance of mammalian tissues requires continuous and discriminative surveillance - detecting and removing any potentially harmful abnormalities whether these arise by somatic mutation or environmental intrusion.

An immunological system responding selectively to foreign antigen has been evolved and may operate by making use of the somatic mutation which itself constitutes a part of the problem. Division of a lymphoid cell with a mutant base sequence in an appropriate part of the genome will produce a clone of cells possessing a highly characteristic (surface) marker - the phenotypic expression of the genetic accident. The characteristic structure of the marker protein allows effective combination with a restricted range of structural (antigenic) determinants. Thus by somatic mutations in one part of the genome an enormous variety of lymphoid cell clones will result capable of reacting with most or all molecules of adequate size. This provides a recognition system. Distinction of 'self' from 'non-self' depends on differences in the response of the lymphoid cell to the presentation of antigen. Either cell death and extinction of the clone may occur, or multiplication and differentiation into antibody producing cells, specific sensitised lymph cells and memory cells (Burnet, 1959).

The sequence of events which does occur depends on a variety of factors, but antigenic concentration, persistence, presentation

and structure are considered to be of particular importance. Thus low doses of antigen, presentation of antigen processed by macrophages and antigen complexed with antibody are alleged to stimulate reactivity rather than paralysis. The reactivity of the lymphoid cell may also be influenced by environmental factors. Large amounts of preformed specific antibody and immuno-depressant drugs both depress reactivity, whereas Freund's complete adjuvant may increase it. On this theory, natural tolerance results from the persistent presence of autoantigen which eliminates autoreactive clones, and also prevents production of low level opsonins which might tip the balance away from paralysis towards reactivity.

This balance is delicate, and errors occasionally occur. Most if not all tissues have been shown to provoke an autoimmune response, and in the tissues and secretions of the reproductive organs alone the list is impressive, viz. spermatozoa (Rumke, 1969; Dukes & Franklin, 1968), seminal plasma proteins (Halpern, Ky & Roberts, 1967), testicular, ovarian, adrenal and placental secretory tissues (Irvine, Chan, Scarth, Kolb, Hartog, Bayliss & Drury, 1968), sex steroids (Beiser, Erlanger, Agate & Lieberman, 1959), gonadotrophins (Rao, 1969) and milk proteins (Bratanov, 1969). Three reasons for the failure to recognise 'self' may be suggested. First, the system whereby antigen suppresses clones of lymphoid cells may break down. Second, antigen which is altered or presented in an atypical manner or

environment may favour immune reactivity rather than tolerance. Third, antigen which is sequestered, present in minute amounts or only for restricted periods may never effectively suppress autoreactive clones and therefore be treated as foreign.

A genetic tendency to develop simple or multiple autoimmune lesions in both mouse and man has been presented as evidence favouring the first suggestion (Burnet & Holmes, 1965; Irvine, 1964). Some evidence also indicates that an autoimmune antibody may be monoclonal, which would be expected if one autoreactive lymphoid cell escaped controls. Even a specific pathological event might not be necessary, for some individuals with autoimmune lesions have an immune system tipped slightly towards a reactive response. For example, men with spermagglutinins tended to produce a fierce response to any antigen (Rumke, 1968). The use of complete adjuvant produces a similar situation experimentally, whilst steroid or ACTH therapy yields the opposite result. Local tissue injury may also have an adjuvant effect, for lesions of the uvea, thyroid, heart and male genital tract are associated with an increase in specific autoimmune reaction (Raitsina & Nilovsky, 1967; Duke-Elder, 1966; Irvine, 1964; Kaplan, 1960; Rumke, 1969).

Local tissue injury may also lead to modification of antigen, for proteolytic degradation of antigen within the leucocytic granuloma induced by Freund's adjuvant has been inferred (Weigle, High & Nakamura, 1969). Immunization with degraded

or altered protein is known to break natural and induced tolerance more readily than intact antigen (Asherson, 1968; Weigle et al, 1969). However, an effect of Freund's adjuvant on lymphoid tissue reactivity rather than antigen is indicated by the finding that autoimmunity to spermatozoa could be induced by injecting adjuvant and testis at different but adjacent sites (Bishop, 1961). The sudden change in dosage caused by the presentation of a foreign antigen which cross-reacts with autologous tissue as occurs in rheumatic fever (Kaplan, 1965) and glomerulonephritis (Dixon, 1968), could tip a stable situation from tolerance to reactivity.

The antigens responsible for experimental allergic encephalomyelitis and autoimmune aspermatogenesis are absent in the neonatal animal. Many other antigens are intracellular or sequestered away from the circulation, and a few such as milk proteins and spermatozoa in the female are present irregularly. Unless cross-reacting antigens are present in other tissues, it is difficult to see how natural tolerance arises. Most if not all these antigens are unlikely to make ready contact with immunologically competant tissues. Thus most spermatozoa are voided in the ejaculate and the urine and some are removed by macrophages within the male tract (Roussel, Stallcup & Austin, 1967). A powerful barrier in the genital tract prevents substantial leakage of free spermatozoal antigen into the systemic circulation. As immunogenic antigen is processed

by dendritic macrophages, the debris removed within the male tract would not be expected to induce an autoimmune response under normal conditions (Nelson, 1968). The occurrence of such a response in men with an inflamed genital tract or following vasectomy can be interpreted as a failure to maintain the barrier intact, coupled with the presentation of a heavy load of debris antigen locally.

The production of an autoimmune reaction to any one tissue will probably incorporate features of all three theories of autoimmunity.

Although one of the first examples of autoimmunity, the reaction against autologous spermatozoa is still very incompletely understood (Metalnikoff, 1900; Adler, 1909). More knowledge of the basis and consequences of the response is important not only for reasons of scientific enquiry, but also for the very practical reason that up to 7% of infertile men and 35% of infertile women reportedly possess autoantibodies to spermatozoa. The presence of the autoantibodies has been causally related to the infertility in some patients, and this has inspired the hope of an contraceptive vaccine. The remainder of this introduction examines the data establishing the antigenicity of spermatozoa, the responsiveness of the immune system to spermatozoal immunogen and the interaction of the response with endogenous antigen. The dissertation describes the results of some investigations into this last phase of autoimmune damage.

## Antigenicity of spermatozoa

The numerous published antigenic analyses of spermatozoa have produced little unequivocal data. Most of the hetero-, iso- and auto-logous antigens described have not been studied in detail either biochemically or immunologically. There is as yet no direct evidence that any one spermatozoal autoantigen is identical with the heteroantigen of the same spermatozoa or whether different molecules are involved. Only infrequently are antigens established as specific to spermatozoa as distinct from those secondarily adsorbed from the genital tract secretions (Weil & Finkler, 1959; Weil & Roberts, 1965). The preparation of purified spermatozoal antigens would help clarify these problems.

The heterologous, isologous and autologous antigens reported as specific to spermatozoa will be briefly reviewed.

(1) Heterologous antigens.

Many analyses have been made of the heteroantigenicity of semen, testis or spermatozoa, but only a few have distinguished spermatozoal antigens from those adsorbed secondarily (Pernot, 1956; Rao & Sadri, 1959, 1960: Matousek, 1964; Hunter & Hafs,

1964, 1965; Maruta & Moyer, 1967). The degree of cross-reaction of spermatozoal antigens with those from other autologous or isologous tissues has established the presence of species, organ and cell specificity (Popivanov & Vulchanov, 1961, 1962b, 1965; Edwards, Ferguson & Coombs, 1964; Tribulev, Podoplelov, Popivanov, Nakov, Zhivkov & Vulchanov, 1967). Most of these studies did not attempt to demonstrate any properties of the heteroantigens such as identity with autoantigens, a specific role in capacitation or fertilization or a specific effect resulting from heteroimmunization. Neither does any reported investigation demonstrate whether a heteroantigen of one species cross-reacts with a sperm autoantigen in the heterologous species. This would be important in the production of a contraceptive vaccine for man, for animal antigen would be easier to obtain and unlikely to contain contaminant autoantigens.

(2) Antigens which are isologous but not autologous.*

The presence of M, N and Tja antigens on human spermatozoa and the absence of Xga has been reported without confirmation (Edwards et al, 1964).

The rhesus antigen D was not detected on spermatozoa by an absorption-elution technique (Levine & Celano, 1961) and by uptake of $I^{131}$-labelled anti-D (Quinlivan & Masouredis, 1962). In contrast, other authors found the antigen using mixed

* The recent description of an Xga (+ve) woman possessing antibodies to Xga antigen may invalidate this distinction (Yokoyama & McCoy, 1968).

agglutination (Gullbring, 1957; Majsky & Hraba, 1960).

The ABO system has been intensively studied with conflicting results. There is general agreement that autologous spermatozoa adsorb A, B and H antigens from the seminal plasma of secretors. Some authors claim that only after such adsorptions are ABO antigens present on spermatozoa (Edwards et al, 1964; Boettcher, 1969; Katszina, Spielman & Trinkaus, 1969) while others claim that the antigens are also an integral part of the spermatozoon and can be detected in non-secretors (Gullbring, 1957; Levine et al, 1961; Popivanov & Vulchanov, 1962a; Shahani & Southam, 1962; Popivanov, Sturkalev, Evrev & Ananiev, 1966; Krieg, 1967; Parish, Carron-Brown & Richards, 1967). Many of these latter authors also claim that in men heterozygous for ABO, haploid expression of blood group genes produces two forms of spermatozoon carrying different ABO antigens. Several reports claim that ABO incompatibility between husband and wife causes infertility but many of the data disregard misidentified paternity, include the effects of incompatibility in pregnancy as well as at fertilization and never approach the theoretical level expected for complete incompatibility. Contradictory data have been produced.

The large epithelial cell contamination of some ejaculates demands cautious interpretation of results produced by absorption techniques e.g. Levine et al (1961), Popivanov et al (1962a), Popivanov et al, (1966), Katszenia et al, (1969).

Immune fluorescence was used to demonstrate the presence of ABO antigens on nonsecretor spermatozoa (Shahani et al, 1962; Krieg, 1967), but failed to detect them on spermatozoa in testis sections in which there was a built in positive control of epithelial cells (Holborow, Brown, Glynn, Hawes, Gresham, O'Brien & Coombs, 1960). Most authors have failed to detect the antigens on spermatozoa by direct agglutination, but some have obtained successful results by the mixed agglutination or mixed antiglobulin tests (Gullbring, 1957; Parish et al, 1967) while others using the same tests have produced contradictory results (Shahani et al, 1962; Edwards et al, 1964). Gullbring did not determine the secretor status of the semen donors, and Parish et al found very weak positive results in only two of four ejaculates tested.

The evidence favouring the presence of ABO antigens on non-secretor spermatozoa is thus very slender. Any antigens that are present must be of low concentration, of restricted distribution or in a position relatively inaccessible to antibody. It is also unlikely that the spermatozoa produced by a man heterozygous for ABO will be of two phenotypes, for there is no evidence for postmeiotic expression in man but good evidence for extensive cytoplasmic connections between developing spermatids.

Recently, Vojtiskova (1969) has claimed that histo-compatibility antigens are present on spermatozoa, but this

claim requires confirmation with a technique which identifies the cell type involved.

(3) Isoantigens which are also autoantigens.

Most studies on autoantigenicity have been done on the guinea-pig, and four spermatozoal autoantigens have been isolated (S,P,T and Z), at least some of which are specific to spermatozoa (Voisin & Toullet, 1968). The first two of these have been prepared in a pure form, and possess between them properties similar to those of the crude testicular extracts prepared by various authors (Freund, Thompson & Lipton, 1955; Brown, Glynn & Holborow, 1963; Brown, Holborow & Glynn, 1965; Kirkpatrick & Katsh, 1964; Bishop & Carlson, 1965). They are also both present in the acrosome, which is known to be potently autoantigenic (Beck, Edwards & Young, 1962; Katsh, 1960). Beck et al described a tail autoantigen in the guinea-pig, but this has not been confirmed. Lewis (1933, 1934) showed that autoantibodies to testis also reacted with other tissues. The testicular antigen(s) involved were not identified.

Human testicular spermatozoa possess autoantigens (Rumke,1969), and the occurrence of at least three agglutination patterns with different autologous antisera suggests as many autoantigens (one acrosomal, one midpiece, one mainpiece).

Specific spermatozoal antigens have not been detected

in the rabbit (Weil et al, 1959; Edwards, 1960b; Beck et al, 1962) and monkey (Moyer and Maruta, 1967). Autoantibodies to spermatozoa from rat, mouse and various other species have been claimed, but have not been proven as directed against spermatozoa rather than adsorbed material. Probably spermatozoal autoantigens occur in any species which can develop autoimmune lesions of the testis (rat, mouse, ram, man and guinea-pig but not rabbit, monkey or opossum - see later).

The induction of an autoimmune response.

The immunological response to any antigen is heterogeneous and the relative proportions of the various components vary with time. As well as the basic division into cellular and humoral responses, the latter may be subdivided and in man eight immunoglobulins have been distinguished antigenically. They are present in normal serum in different proportions (Cohen & Milstein, 1967), can be induced differentially (Yount, Dorner, Kunkel & Kabat, 1968) and possess different immunological properties (Terry 1965; Ishizaka, Ishizaka, Salmon & Fudenberg, 1966). Any one immunoglobulin may possess several immunological properties, thus guinea-pig gamma$_2$-Globulin fixes complement, causes the Arthus response and is both cytotoxic and

cytophilic. The specific sites endowing these properties are located on the heavy chain.

The nature of the antigen, its dose and the route and method of its presentation have all been shown to affect the types and sequence of responses. For example, one spermatozoal extract induced a strong cellular reaction with no humoral response whilst administration of another extract in incomplete adjuvant induced $gamma_1$-globulin but prevented development of both cellular and $gamma_2$-globulin responses (Brown, Glynn & Holborow, 1967). Autoantigen S evoked delayed-hypersensitivity by day 7, anaphylactic reactions by day 10 and Arthus reactions by day 14 in the guinea-pig. An equivalent dose of autoantigen P evoked these same reactions by day 21, day 14 and 21 respectively (Voisin & Toullet, 1969). Glycoproteins like S may be more readily retained and this may affect the immune response (Eyquem, 1969). A systematic study of the types of immune response resulting from immunization with pure spermatozoal antigens may be of practical importance, for PCA antibodies protect spermatozoa and testicular cells *in vitro* from damage by spermatoxic antibodies (Chutna & Rychlikova, 1964). If this occurred *in vivo*, establishment of a high and early titre of PCA antibodies should prevent damage, whilst a low titre would facilitate it.

The nature of the antigen will also determine which of the several properties of each immunoglobulin type will be expressed.

Thus, guinea-pig autoantigen P induces antibodies that precipitate and fix complement but are not cytotoxic, whereas autoantigen T induces both spermatoxic and complement-fixing antibodies (Voisin et al, 1968). This suggests that P is a soluble divalent antigen which is probably not on the cell surface, whereas T is an insoluble surface bound antigen. This inference has been fully supported by physico-chemical tests. The relevance of these in vitro tests to what occurs in vivo is unresolved.

The route of administration of antigen also affects the immune response. Local application of antigen can induce a local response. Rumke (1969) has detected higher antispermatozoal titres in the seminal plasma than in the serum of some infertile men; local synthesis provides one explanation for this finding. Local antibody synthesis to semen antigens does occur in the cervix and vagina of guinea-pig, rabbit and woman (Ashitaka, Isojima & Ukita, 1964; Edwards, 1960b; Parish et al, 1967; Parsons & Hyde, 1940: Straus, 1965). Uterine antibody synthesis is less well established, due to the difficulties in bypassing the vagina and cervix without traumatising the uterine endometrium. Local resistance to uterine infection does occur (Edwards, 1969), but attempts to induce uterine antibodies to semen have not been successful (Sokolovskaya & Reshetnikova, 1968; McClaren, 1966).

The production of autoimmune lesions.

Production of an immune response to autologous or isologous spermatozoal antigens does not necessarily cause infertility. However, several conditions of impaired fertility may be observed. In the male, autoimmune reactions may depress the formation of spermatozoa or agglutinate them in seminal plasma. In the female, the transport and fertilizing capacity of spermatozoa may be reduced or the foetus damaged in early pregnancy.

(1) Autoimmune aspermatogenesis.

This condition was first described in the guinea-pig (Voisin, Delaunay & Barber, 1951; Freund, Lipton & Thompson, 1953).but has also been produced in mouse (Pokorna, Vojtiskova, Rychlikova & Chutna, 1963; Shetye & Rao, 1968), rat (Freund, Lipton & Thompson, 1954; Katsh & Bishop, 1958; Imbabi, 1967), ram (Busey, 1965) and man (Mancini, Andrada, Saraceni, Bachmann, Lavieri & Nemirovsky, 1965). Lesions were not induced in the rabbit, opossum and monkey (Tyler & Bishop, 1963). Two problems have dominated discussions on autoimmune testicular lesions: the type of immune response which causes the damage and specificity of damage.

It has widely been accepted that the testicular lesions are caused by a delayed-type hypersensitivity response involving immune cells. Four pieces of evidence have been quoted in favour

of this, but none of them are very convincing. Thus, complete adjuvant was at first found necessary to elicit damage, and it is known that adjuvant boosts the production of delayed-hypersensitivity. However, adjuvant also affects the type and sequence of antibody production and increases the immune response generally. Damage has also been produced without adjuvant (Bishop, 1961; personal observation).

Secondly, autoimmune lesions have been transferred by immune cells (Laurence, 1962; Imbabi, 1967; Stone, Lerner & Goode, 1968), but attempts to transfer damage by serum have proved unsuccessful (Bishop *et al*, 1965). The use of cell transfer as unequivocal proof of a cell-mediated damage alone is open to doubt. The possibility that the transfer may include antibody cytophilic for transferred cells (Jonas, Gurner, Nelson & Coombs, 1965), specific immune cells capable of synthesising antibodies (Paterson, 1966) or small quantities of highly immunogenic antigen (Askonas & Rhodes, 1965a,b) necessitate rigorous controls, which have not been reported.

Thirdly, the occurrence of autoimmune damage is alleged to show a better correlation with the presence of delayed hypersensitivity than humoral antibodies. However, in no published studies is there a complete correlation with delayed hypersensitivity, nor a complete analysis of all types of antibody response. The most potent damaging antibodies in autoimmune thyroiditis and glomerulonephritis are removed *in vivo* by the

target tissues (Lerner & Dixon, 1966; Nakamura & Weigle, 1969). A detailed investigation by Voisin and Toullet (1968) found that of those males showing aspermatogenesis 9% had a delayed hypersensitivity response only, 26% had a humoral response only, and 67% had both types of response. The same authors (1969) failed to detect autoimmune lesions when a delayed-response only was present, but damage occurred with the appearance of antibodies capable of giving an anaphylactic or Arthus response. Brown et al (1967) have reported similar results.

Finally, some authors have found a cellular infiltration of the testis (Voisin et al, 1961; Freund et al, 1955; Mancini et al, 1965; Waksman, 1959; Raitsina et al, 1966; Voisin, 1969) whilst others either deny this (Freund et al, 1953; Katsh, 1960; Brown et al, 1967; Imbabi, 1967) or fail to describe it (Katsh et al, 1958; Katsh, 1959, 1964; Vojtiskova, Chutna, Rychlikova & Pokorna, 1962; Pokorna et al, 1963; Chutna & Rychlikova, 1964; Bishop et al, 1965; Shetye et al, 1968). Most of the authors failing to detect cellular infiltration sampled testes late after immunization, whereas those detecting it examined testes earlier. A notable exception is that of Brown et al (1967) who sampled serially from day 7 onwards and detected no cellular inflammation. The cells at the site of lesions are usually described as mononuclear, histiocyte or round cells with some neutrophils and the histology of the 24 hour skin reaction was very similar to that of the testis (Mancini

et al, 1965). Unfortunately, the occurrence of a cellular infiltrate is not conclusive evidence in favour of "cellular damage". Antigen-antibody interactions, with or without delayed-hypersensitivity reactions, will attract leucocytic cells and no absolutely characteristic pattern exists. However, the cellular infiltrates described by the above authors are suggestive of a 'cellular' component of damage, possibly coupled with damage by antibody.

Evidence which conclusively distinguishes between cellular, humoral or combined reaction as the cause of testicular damage is thus lacking. The possibility of interaction between the types of humoral response makes the discovery of such evidence more difficult. The search could, however, be a false one, for all three mechanisms might actually operate in appropriate circumstances.

The specificity of the immune response to spermatozoa has been convincingly demonstrated, but several authors have queried the specificity of testicular damage resulting from isoimmunization with testis, suggesting that damage results either from hormonal disbalance or local nonspecific damage in or near the testis.

Autoantibodies to testicular secretory tissue, steroid hormones and gonadotrophins have been detected (Irvine et al, 1968; Lieberman, Erlanger, Beiser & Agate, 1959; Ferin, Zimmering, Lieberman & Vander Wiele, 1968) but although the presence of such antibodies following isoimmunization with testis has not been

investigated, it seems unlikely that they are the cause of testicular damage. Thus, injection of isologous spermatozoa either intact or as highly purified extracts produces similar lesions to those with whole testis (Freund et al, 1955; Voisin et al, 1968, 1969). Secondly, immunization of guinea-pigs with testes in which only germ cells are absent does not induce aspermatogenesis (Katsh, 1960). Thirdly, no changes in the size, function or histology of the androgen dependant accessory tissues nor in the properties of their secretions have been detected during aspermatogenesis despite intensive study by several authors (Freund et al, 1955; Katsh et al, 1958; Mancini et al, 1965; Imbabi, 1967). Fourthly, species in which spermatozoa are not autoantigenic do not develop autoimmune lesions of the testis. Finally, damage is initiated at the rete testis and not at the site of secretory tissue (see later).

A single injection of adjuvant in or near the perineal region caused testicular inflammation within one day, and any latent infection exacerbated this process (Voisin et al, 1951). However, when two or three injections of tissue in adjuvant are made in the back, there is great specificity of damage. Of many homologous tissues tested only brain and testis regularly produce testicular lesions at low or moderate doses (Freund et al, 1953; Katsh et al, 1958; Bishop et al, 1965). Recently Vulchanov (1969) has described aspermatogenesis in the guinea-pig following several injections of homologous seminal vesicle fluid in adjuvant into the hindfoot

pad. The animals did not react immunologically with testis antigen, and the specificity of damage was questionable. Production of testis damage by repeated injections of heterologous testis or spermatozoa was reported by Katsh et al (1958), but in the absence of any serological data the specificity of this response also remains doubtful.

Spermatogenesis is a sensitive process easily disturbed by non-specific trauma. In the induction of specific immune aspermatogenesis purified antigen should be used, only one or two injections in a minimum of adjuvant should be given at a site remote from the testis, any animals showing ill health should be discarded, skin and serum responses to spermatozoa should be detectable and features of testicular damage characteristic to autoimmune lesions should be identified.

(2) Immune reactions in the male which do not cause aspermatogenesis but which do cause infertility.

Only in man has an antifertility effect against seminal spermatozoa been reported (Wilson, 1954; Rumke, 1954). The seminal plasma and serum of these men contained a factor which agglutinated autologous or isologous spermatozoa, and prevented penetration of cervical mucus (Fjallbrandt, 1968b). The presence of serum spermagglutinis was not invariably associated with infertility, even when titres were as high as $^{1}/_{1024}$ (Phadke

& Padukone, 1964; Fjallbrandt, 1967, 1968a), although the incidence of high titres was greater in the sterile group compared with the fertile group. The dissociation of serum titres from infertility could be due to any of several factors. Thus IgM agglutinins may transmit to seminal plasma less efficiently because of their greater molecule size. Different classes of immunoglobulin are characterised by specific loci on the heavy chain, which is also the site determining transmissability across membranes (Brambell, Hemmings, Oakley & Porter, 1960). Agglutination by readily transmittable immunoglobulin would probably be associated with greater infertility. Also, serum agglutinins may not be antibodies but some non-specific factor which does not affect the ejaculate.

Clinical histories of many of the men with spermagglutinins frequently contain evidence of inflammation in the genital tract, suggesting a cause of primary immunization. Therapy for such men is not currently available, although Schoysman (1968) has reported successes with the use of testosterone to temporarily depress spermatogenesis and remove antigenic stimulation. A fertile ejaculate reappeared before high titres of spermagglutinins.

(3) Antibodies to spermatozoa in the female.

The sera of up to 25% of infertile women and 72% of

prostitutes allegedly contain spermagglutinins (Dukes et al, 1968; Schwimmer, Ustay & Behrman, 1967). Sexual abstinence ~~of~~ or use of condoms reduced both the titres of spermagglutinins and the infertility. The serum spermagglutinins were not characterised as antibodies, and other workers have not found such a high incidence of agglutinins (Tyler, Tyler & Denny, 1967; Israelstam, 1969).

Studies on experimental animals have been equally conflicting, although most authors have reported some impairment of fertility by systemic immunization of females with spermatozoa or testis. Results fall into two groups - those showing marginal and those showing major effects.

The fertility of mice isoimmunized with epididymal spermatozoa was slightly reduced probably due to lower fertilization rates (Edwards, 1964). The fertility of rabbits immunized with seminal coating antigen, and of goats immunized with spermatozoa or seminal plasma, was unaffected despite the appearance of antibodies (Weil et al, 1965; Goel, Soni & Bhalla, 1967).

In contrast repeated intraperitoneal injections of spermatozoa into mice induced substantial infertility by lowering the fertilization rate. The massive deposition of spermatozoal enzymes into the peritoneal cavity could cause peritonitis or damage to ovary and ovulated eggs, and no search for any such inflammation is reported (McLaren, 1964, 1966). Several workers

have depressed fertility in the guinea-pig by 40-80% as judged by the number of live young produced (Isojima, Graham & Graham, 1958; Behrman & Otani, 1963; Katsh, 1959; Plank, 1967). Frequently, multiple injections in adjuvant were used, and many animals were suffering from amyloidosis in one study (Plank, 1967).

A reduced bovine fertility and fertilization rate related to antisperm antibody titre has been reported (Menge, 1967; Sokolovskaya, 1967; Sokolovskaya et al, 1968). A complete infertility was induced in rabbits immunized with ejaculated spermatozoa and the infertility appeared when antibody titres were high, but was maintained when they fell (Behrman & Nakayama, 1965). The antibody was directed against a sperm coating antigen and this result contradicts that of Weil et al (1965).

In the above studies, one or more antigens were present in the immunizing dose of testis, seminal plasma or spermatozoa and not surprisingly this has produced very variable results. Purification of antigens and use of these might clear some confusion and help to establish any relationship between antibody titre and infertility that exists. There was also great variation in the time after immunization at which the assessment of infertility was made. Behrman et al (1965) followed the titre of antibodies and did test matings at intervals during the primary and secondary responses. They could correlate infertility with titres. Most other workers mated at some random intervals after immunization, or even attempted repeated matings over a period of

weeks. This is bound to affect the assessment of the degree of infertility.

Some or all of the antifertility effect seems to be exerted on spermatozoa prior to fertilization; however, there are claims that antisperm antibodies increase embryonic mortality (Katsh, 1959; Menge, 1967, 1968, 1969; Sokolovskaya et al, 1968, 1969), and any study in which crude birthrate is used as a measure of reduced fertility will include such an effect. These claims imply that sperm antigens are present in the foetus in sufficient amount to cause foetal death following damage by antibodies or immune cells. These antigens are presumably not present, accessible or vulnerable in the mother. Spermatozoal isoantigens are not present in the prenatal animal, although the brain antigen cross-reacting with testis might be (Katsh et al, 1958). However, this antigen, like that inducing allergic encephalomyelitis may only appear after the blood-brain barrier develops (Paterson, 1958). Menge & Protzman (1967) found cross-reacting heteroantigens in bovine conceptus material and spermatozoa, but Isojima & Li (1968) could not confirm this in the rat.

Testis and semen injections would immunize the mother against histocompatibility antigens, which are also present on the embryos (Simmons & Russell, 1966). Embryonic or foetal damage might then result. The use of pure antigens would clarify this problem. Also, a comparison of the survival rates of blastocysts transferred to the kidney capsule of male mice auto- or iso-

immunized with testis would differentiate the anti-blastocyst activity caused by antibodies to spermatozoa from that caused by histocompatibility antibodies.

Despite the confusion produced by the results of these experiments, several points emerge. (1) spermatozoa are autoantigenic in the female; (2) immunization with spermatozoa can impair fertility; (3) at least part of the antisperm activity acts to reduce successful fertilization.

# Chapter 1

## A NATURALLY-OCCURRING ANTIBODY TO SPERMATOZOA

## INTRODUCTION

Metchnikov in 1900 noticed that normal fresh serum from some species was spermicidal. The involvement of complement was suggested by several authors (Walsh, 1925; Lumsden, 1929; Chang, 1947; Drevius, 1968), and ~~this~~ was later demonstrated using the specific immunological tests of complement fixation, mixed conglutination and immune adherence (Weil et al, 1959; Edwards, 1960a; Lachmann, Sell & Spooner, 1965; Edwards, 1967). Several of these tests have shown that prespermatozoal cells react with a similar or identical serum factor (Spooner, 1964; Lachmann et al, 1965).

Complement can cause lysis of blood cells without an antigen-antibody reaction (Muller-Eberhard & Lepow, 1965), but a similar explanation is unlikely to apply to the lysis of spermatozoa by normal serum for the reaction can also be detected by head-to-head agglutination, the mixed antiglobulin test and immune fluorescence using heat inactivated sera (Beck et al, 1962; Otani & Behrman, 1963; Matousek, 1964; McLaren, 1964; Edwards, 1967; Menge, 1967; Symons, 1967). Also, using diluted fresh serum as a source of complement, lysis of spermatozoa occurs on addition of inactivated normal serum (Drevius, 1968). Another serum factor appears to react with the spermatozoal acrosome and in doing so fixes complement, which may cause lysis.

Few tests to determine the nature of this factor have been reported. It is present at reduced levels in the sera of young

animals (Edwards, 1960; Beck et al, 1962) and is specifically directed against the spermatozoon and its precursor cells (Edwards, 1960; Spooner, 1964; Monastirsky & Fernandez Collazo, 1967). The activity is often not species specific, and in some species, including man, is absent or of very low titre.

This chapter describes the properties of this antibody-like activity in the guinea-pig.

MATERIALS AND METHODS

Immune fluorescence

The globulin fractions of rabbit antisera to guinea-pig globulin, gamma-globulin and albumin were conjugated with fluorescein and absorbed with guinea-pig spermatozoa and liver powder (Nairn, 1964). Fluorescein-conjugated antiserum to $\beta_{1C}$ was kindly provided by Dr. P.J. Lachmann.

The indirect immune fluorescence procedure was used. Spermatozoa from the epididymis or vas deferens were smeared on to circular glass areas of otherwise siliconed slides, and were fixed by air drying, acetone or 80% ethanol. Testes were fixed in cold ethanol, paraffin embedded, sectioned and the sections placed on slides as described by Sainte-Marie (1962). The smears or sections were incubated with serum in a humid chamber at 37°C for 30 minutes, washed for 30 minutes with phosphate buffer (pH 7.1), incubated with conjugated antiserum for 30 minutes and washed again. They were then mounted in buffered glycerol, coded and examined with a Zeiss phase-fluorescent photomicroscope using dark-field illumination (Super pressure mercury vapour lamp HBO 200W, exciter filters BG3 and BG38, barrier filter 47). The control smears and sections indicated in table 1 were coded and randomly mixed with test slides for scoring.

The effect of pH on the stability of the antigen-antibody interaction was tested by the use of the first washing buffer at

the desired pH, followed by a five minute rinse in phosphate buffer (pH 7.1) before incubation with conjugated antiserum.

Ultracentrifugation

0.5 ml. of cleared guinea-pig serum was centrifuged on 10-40% sucrose gradient for 4½ hours at 64,000 r.p.m. Fractions were collected, analysed for protein and those in two major peaks were pooled, dialysed and concentrated.

Sephadex gel Filtration

5 ml. of guinea-pig serum were dialysed against tris-HCl buffer (pH 8.0) and applied to a Sephadex G-200 column (4 x 48 cm). The fractions were eluted with tris-HCl buffer (pH 8.0) at 35 ml/hour and collected on an LKB RadiRac collector. Fractions were measured for protein content by passage through the LKB Uvichord Densitometer. Peaks were concentrated, dialysed and tested immunoelectrophoretically.

Ion-exchange chromatography

The two-step elution method of Reisfeld & Hyslop (1966) was used for ion exchange chromatography. 12 ml. of guinea-pig serum were dialysed against phosphate buffer (pH 7.9, 0.009M $K_2HPO_4$, 0.001M $KH_2PO_4$) and applied to a column 1 x 31 cm. of Whatman DE52 equilibrated with the buffer. The $gamma_2$-globulin was eluted with the low molarity buffer at 50 ml/hour. The high molarity

buffer was then applied (pH 7.9, 0.282M $K_2PO_4$, 0.018M $KH_2PO_4$) and the fraction containing $gamma_1$-globulin eluted.

Passive cutaneous anaphylaxis (PCA)

Guinea-pigs (250-300 gm.) were injected intradermally with 0.1 ml. of normal guinea-pig serum, 0.1 ml. of saline (negative control) and 0.1 ml. of guinea-pig antiserum induced to homologous epididymal spermatozoa (positive control). Five hours later 0.5 ml. of 2% Geigy-Blue B was injected intravenously followed by 0.5 ml. of the supernatant of a guinea-pig testis homogenate in saline (1000 mgm/ml.). Intradermal sites were examined for bluing after fifteen minutes

RESULTS

Properties of the serum factor

Normal guinea-pig serum gave a brilliant acrosomal fluorescence that was detectable in most sera to a titre of 1:8 to 1:16 and in a few to 1:64 (figure 1 and table 1). Spermatozoa devoid of the acrosome did not show fluorescence. Fixation of the antigen with alcohol or acetone did not diminish the fluorescence, which was absent however after fixation by formalin. 1 ml. aliquots of two guinea-pig sera were absorbed with 0.15 ml. volumes of guinea-pig brain, spleen, liver, testis, spermatozoa or kidney prepared by homogenising equal weights of each tissue in 10 mls. of saline. The sera were centrifuged and tested for activity (table 2). Testis and spermatozoa were most effective at removing the antibody. However, 0.5 ml. aliquots of all the tissue homogenates removed most of the activity, and the results of absorption studies on serum with weak activity must be interpreted with caution.

The production of acrosomal fluorescence was optimal in the pH range 5-8 (figure 2) and at 37°C, but was poor at 4°C. The serum factor was destroyed by heating for 30 minutes at temperatures exceeding 68°C, was precipitated by 50% ammonium sulphate but not by dialysis against distilled water. It was not destroyed by 0.2M or 0.4M mercaptoethanol, but weakened by mercaptoethanol followed by iodoacetate.

These properties indicated that the factor was a protein, and the serum proteins were therefore fractionated and the distribution of activity in the fractions tested.

Immunochemical identification of the serum factor

After ultracentrifugation of guinea-pig serum, all activity was in the 7S peak. This was confirmed by gel filtration on Sephadex G-200, following which activity was present in the second peak only (figures 3 and 4). Symons (personal communication) has recently obtained the same result. Ion exchange chromatography on DEAE Cellulose separated an immunoelectrophoretically pure gamma-globulin fraction which possessed strong antispermatozoal activity (figures 3 and 4).

Immunological properties of the serum factor.

The antispermatozoal activity of normal serum was clearly associated with the $gamma_2$-globulin fraction. This fraction contains antibodies which fix complement, are cytotoxic, cytophilic and induce an Arthus reaction, whereas the $gamma_1$-globulin fraction elicits PCA in the guinea-pig (Bloch, Kourilsky, Ovary and Benacerraf, 1963; Jonas et al, 1965). The natural antibody should therefore fix complement but not elicit PCA. These expected properties were tested experimentally.

(1) Spermotoxicity, immune adherence, mixed conglutination and complement fixation are properties of the antibody already

reported. Two further experiments support this finding of complement involvement.

(a) Smears of spermatozoa were incubated with fresh normal serum, aged serum, serum heated at 56°C for 60 minutes to remove $C'_1$ and $C'_2$ or serum treated with $NH_4OH$ to remove $C'_4$ and $C'_3$. After washing, smears were incubated with fluorescein-conjugated anti-$\beta_{1C}$. Only spermatozoa treated with fresh serum showed acrosomal fluorescence.

(b) Lysis of guinea-pig spermatozoa was observed by phase microscopy using various dilutions of normal fresh serum. The lysis and immobilisation of spermatozoa started within five minutes of adding the serum, the first sign of damage being vesiculation of the acrosome. This was followed by rupture of the acrosomal membranes, an increased eosin permeability and a subsequent drop in motility. The acrosomal damage thus precedes the immobilisation. This observation confirms that the antigen is present in the acrosome, a conclusion also reached by immune fluorescence, agglutination and the mixed antiglobulin test.

(2) 40 normal sera were tested for their ability to elicit PCA. None of these gave a positive result, in the presence of vivid positive oontrols, demonstrating that the antibody does not possess this property.

Reaction between normal serum and germinal cells in the testis

The reactivity of normal serum with germinal cells was

tested by applying the indirect immune fluorescence procedure to testis sections. Antisera induced to homologous spermatozoa produced strong fluorescence of the acrosomal area of spermatozoa and spermatids. With normal serum an identical pattern was observed, although the fluorescence was fainter and of low titre.

The staining pattern in any tubule was characteristic for the stage of spermatogenesis of the tubule. Staining appeared first as a small cone in the round-oval spermatids of stage 6, and increased to cover the whole acrosome. Although staining could not be correlated precisely with a specific cell structure in the phase contrast observations, the area presumably represents that part of the cell membrane which forms the outer acrosomal membrane. This part is structurally and physiologically distinct from the remainder of the surface membrane of the spermatozoon (Quinn, White & Cleland, 1968).

## DISCUSSION

Naturally occurring antibodies to a variety of antigens, and in particular bacteria, have frequently been reported (see Boyden, 1966). They are often described as thermolabile macroglobulins although this generalisation has many exceptions. Thus, recently natural antibodies to two bacteria have been characterised as gammaG-globulins (Beernink & Steward, 1968; Spitznägel, 1966).

There are fewer reports of naturally occurring antibodies to autologous and homologous antigens. Kidd & Friedewald (1942) described a factor present in fresh rabbit serum which reacted with saline extracts of various homologous tissues (kidney, liver, spleen, brain, testicle). It was complement-fixing, precipitated by ammonium sulphate, destroyed by heating to 65°C and its levels were low in young rabbit sera. This natural antibody reacted with an intracellular organelle and not with intact cells, but the lysosomal origin of the acrosome could mean that the same factor is involved in the spermotoxicity of normal serum. The presence of naturally occurring kidney antibodies in the normal sera of the rabbit has been described (Spar, Bale, Wolfe & Goodland, 1956). A heat stable serum factor reacting with homologous liver and identified as IgA or IgM was obtained from the serum of 70% of normal rats (Weir, Pinckard, Elson & Suckling, 1966).

The presence of natural antibodies to homologous antigens is becoming accepted, but their origin and significance is less certain.

The origin of the natural antibody

Sera from males and virgin females possess the natural antibody to spermatozoa which cannot therefore result from autoimmunization with spermatozoa. The 'natural' antigen may cross react with common (bacterial) antigens to which all animals are exposed, but Symons (personal communication) was able to detect the antibody in sera from 'germ free' mice. The possible presence of dead bacteria or antigens in the food reduces the value of these studies. Absorption of guinea-pig serum with sheep red blood cells does not significantly reduce the titre and intensity of fluorescence, indicating an absence of anti-Forssman activity by the antibody. Drevius (1968) dissociated the spermicidal activity of fresh rabbit serum from the sheep red blood cell haemolysin.

The alternative to a low-specificity antibody is an antibody produced under genetic control, possibly as a necessary component of the immune recognition system. If this was the case, then natural antibodies should occur to all potentially antigenic material, including all autoantigens. However, proof of the existence of this system is difficult to obtain, for failure to detect a natural antibody to a known antigen could merely reflect the insensitivity or incorrectness of the immunological test used. Also natural antibodies may represent 'over flow' of the specific antibody-like markers on the immunologically competent lymphocyte.

The function of the natural antibody

(1) The antibody could form part of the immune recognition system, as suggested by Jerne (1955). The isolation of four spermatozoal autoantigens would demand four natural antibodies (Voisin et al, 1968). As at least two of the autoantigens appear to be acrosomal, the natural antibody characterised above could be a complex of antibodies. The use of pure antigens would elucidate this problem.

(2) Natural antibodies may behave as opsonins. The attachment of cellular antigens to macrophages is non-specific, but ingestion is facilitated by the presence of antibody (Vaughn & Boyden, 1964), and several authors report that IgG is more efficient than IgM at opsonisation in vitro (Smith, Barnett, May & Sandford, 1967; Rabinovitch, 1967a,b). There is also evidence for the involvement of serum factors in the ingestion of 'worn-out' autologous tissues (see Shands, 1967). Many of the antigen-antibody systems studied for their ability to elicit a phagocytic response have involved complement (Nelson & Lebrun, 1956; Boyden, 1962 a,b) which is also fixed by the natural antibody to spermatozoa.

Spermatozoa are readily phagocytosed in vivo in the female and male tracts and the process is initiated at the acrosome in normal mice (Symons, personal communication), hamsters (Bavister, personal communication) and rabbits (Bedford, 1965). Live homologous spermatozoa also adhere by the acrosome to macrophages

taken from the peritoneal cavity of the guinea-pig (personal observation). The possibility that the gamma$_2$-natural antibody has attachment sites cytophilic for macrophages cannot be excluded.

Phagocytosis of antigen-antibody complex could lead to an enhanced immune response but may also be a method of sequestering material outside the immune system and digesting the antigens. The phagocytic cell type may be important here for whereas engulfment of antigens by lymphoid tissue macrophages enhanced their immunogenicity, polymorphs and scavenger macrophages were more concerned with removing and excreting foreign materials (Askonas et al, 1965a; Nelson, 1968). Effective disposal of redundant spermatozoa is very necessary in both the male and female, and the relatively low incidence of induced autoantibodies demonstrates the efficacy of the system. Whether or not the natural antibody is involved remains uncertain. Symons (1967) could not demonstrate the antibody in the male tract but a serum globulin resembling the natural antibody was present on dead spermatozoa only flushed from the uteri of mated mice.

In view of the apparent absence of circulating natural antibodies to spermatozoa in some species, including humans, a role for the antibody in any process essential for promotion or depression of an immune response to spermatozoa seems doubtful.

(3) The natural antibody may be involved in capacitation in some species. The acrosomal changes which can be seen by

phase or electron microscopy following addition of fresh serum to spermatozoa very closely resemble the 'acrosome reaction' of spermatozoa capacitated *in utero* (Bedford, 1969). Capacitation seems to involve factors associated with follicular fluid (Barros & Austin, 1967; Yanagimachi, 1969) and this fluid is rich in serum protein including gamma-globulin (see later). Bavister (personal communication) has tested the effectiveness of normal serum in capacitation of spermatozoa, but despite the presence of an 'acrosome reaction' failed to get *in vitro* fertilization. This does not eliminate the natural antibody as part of the capacitatory mechanism, for the timing of the reaction and its relation to other synergistic, antagonistic or sequential factors need investigating further.

(4) The natural antibody may have arisen fortuitously as a result of cross reaction with other common antigens, and might therefore have no specific function. Its presence is apparently quite compatible with fertility, and the antibody must somehow be restrained from exerting its cytotoxic effects in the genital tracts. The presence of barriers preventing free movement of antibodies into these tracts could exercise the restraint. If this was so, then boosting the level of serum antibody by an induced immune response to spermatozoa, or disrupting the barriers in the immunized or unimmunized animal should result in damage. The remaining chapters describe some experiments which suggest these immunological barriers in the

genital tracts do exist and that changes in their properties can have important pathological consequences.

## Chapter 2

# AN IMMUNOLOGICAL BARRIER IN THE TESTIS

INTRODUCTION

The use of several techniques has shown that the testis possesses a barrier to free diffusion which has both vascular and tubular components.

The tracing of marker molecules in the testis has proved especially valuable. Acriflavine, trypan blue, light green and Niagara Sky Blue B do not readily leave testicular blood vessels whereas L-dopa and dopamine rapidly equilibrate with intertubular tissue. None of these substances penetrates into tubules (Waksmann, 1960; Kormano, 1967a,b, 1968; Kormano & Pentilla, 1968). Preliminary observations on isolated seminiferous tubules with a central segment bathed in a solution of acriflavine indicate that the dye will stain peritubular but not intratubular nuclei. This clearly locates a component of the barrier at the tubular level, a conclusion confirmed by the observation that the large molecules of the protein peroxidase cannot be detected histochemically within the tubules after injection interstitially (Heidger, 1969).

Secondly, the diffusional space within the testis is not identical when measured by different radioactively labelled substances. At least three extracellular compartments are suggested, a small albumin space, a medium rubidium space and a large iodoantipyrine space (Waites & Setchell, 1966). The rubidium and iodoantpyrine spaces become identical following destruction of the seminiferous tubules (Setchell, Voglmayr

& Waites, 1969).

Thirdly, the compositions of testicular blood and lymph are similar to each other but are very different from that of rete testis fluid. The estimations do not give precise information about the efficiency of the barriers because the seminiferous tubule fluid may be modified in the rete testis and because the lymph drains mainly from the interstitium of the rete testis and the caput epididymidis. However, the results indicate that the tubular barrier is much more effective than the vascular barrier (Cowie, Lascelles & Wallace, 1964; Setchell, 1967). Analysis of the kinetics of diffusion of radioactively labelled substances into rete testis fluid and testicular lymph during constant perfusion into serum provides further supporting evidence for a two-stage barrier with a weak vascular component (Setchell et al, 1969).

Finally during the development of the barrier in the neonatal rat (Kormano, 1967a) and during its breakdown after cadmium administration (Kormano, 1968; Johnson, 1969) there is evidence of two phases of permeability change, one related to a vascular component and the other to a tubular component of the barrier.

The weak vascular barrier is presumably located in the endothelial cells of the A-1-$\alpha$ type testicular capillaries (Crabo, 1963; Clegg & Carr, 1967), which are similar to those in the brain but unlike the capillaries around the ductuli

efferentes and rete testis which possess apertures in their endothelia (Crabo, 1963; Ross, 1963; Murakami, 1966; Bennett, Luft & Hampton, 1959; Leeson, 1962). The recent observation that postcapillary venules are the major sites of fluid exchange may mean that solute transfer is also maximal there (Wiederhielm, 1966). Waksman (1960) has correlated venular permeability with the effectiveness of the immunological barrier at a variety of sites.

The precise location of the tubular barrier is uncertain. The tubule membrane is composed of four layers, an inner acellular layer (Layer 1), a layer of thin overlapping myoid cells (2), an outer acellular layer (3) and an outer loose cellular layer (4) (Lacy, 1967; Ross, 1967). The Sertoli cells rest on layer 1 and are connected by tight junctions (Flickinger & Fawcett, 1967). Acriflavine stains the nuclei of the thin myoid cells but not the more luminal nuclei (personal observation). The barrier is therefore probably located in the luminal membrane of the myoid cell, in acellular layer 1 or in the outer membrane of the Sertoli cell. More precise localization of the barrier should be obtainable by combining tracer or histochemical techniques with electron microscopy of the testis.

The barrier at the rete testis is less effective. Dyes and labelled albumin enter the rete testis but not the seminiferous tubule (Waksman, 1960: Kormano, 1967a) and acriflavine injected into rams with cannulated vasa efferentia stains the spermatozoa

carried out by rete testis fluid before any staining of spermatozoa can be detected in the seminiferous tubule (unpublished work with B.P. Setchell).

There is some evidence that the testis barrier isolates the contents of the seminiferous tubules immunologically. Examination of rat and guinea-pig testis following either intravenous injection of fluorescein-labelled globulin or direct application of fluorescein-labelled anti gamma-globulin to fresh sections revealed no intratubular fluorescence (Mancini, Vilar, Alvarez & Sieguer, 1965; Raj Gupta, Barnes & Skelton, 1967; figure 37).

In this chapter, data on the quantitative transfer of antibody into the seminiferous tubule are presented.

MATERIALS AND METHODS

Collection of rete testis fluid of ram

The cannula was inserted into the ductuli efferentes of the ram as described by Voglmyr, Scott, Setchell & Waites (1967). The caput epididymidis (figure, 5) was separated from the testis along the fascial plane between them, exposing the short ductuli efferentes (figure, 6), which were cannulated with a T-piece cannula (figure, 7). The rete testis fluid passed up the shaft of the T-piece under secretion pressure and was sucked into the collecting vessel by a continuous cross-suction across the bar of the T-piece. The caput epididymidis was replaced over the cannula, which remained in place and functional for up to a month postoperatively (figure, 8).

Protein analysis

Protein concentration was analysed by the Biuret technique on the Unicam spectrophotometer SP500. Bovine serum albumin was used to produce a calibration curve.

Concentration of protein solutions

Two techniques were used. Colloidin membranes of controlled porosity were made as described by Adair (1961), and used for ultrafiltration. Alternatively, solutions were dialysed against 0.004M phosphate buffer (pH 7.1) and lyophilised.

Total and selective protein losses by these techniques were shown to be negligible by comparing cellulose acetate traces of normal ram serum with those for ram serum reconcentrated after dilution 500 fold with saline.

Simple radial immunodiffusion

A 2% solution of agar in barbiturate buffer (pH 8.6) was mixed with antiserum to sheep light chain in the ratio 9 volumes to one volume, poured evenly on to Petri dishes and allowed to gel. 1/1 volumes of the test and standard solutions were placed in holes cut in the gel and left to diffuse radially for two days. Plates were washed, stained and the area of precipitation measured. A standard curve was constructed using ram gamma$_2$-globulin. The technique could detect as little as 0.1 mg/ml. of immunoglobulin.

Cellulose acetate electrophoresis

The phoroslide electrophoresis apparatus (Millipore) was used. Strips were stained and cleared, and the protein distribution analysed on the Joyce-Loebl Chromoscan.

Immunoelectrophoresis

The microtechnique of Scheidegger (1955) was used.

RESULTS

The protein and immunoglobulin content of ram testis fluid

The rete testis fluid was cleared of spermatozoa by centrifugation and Millipore filtration (0.22 filter, GSWP). Samples of the fluid were concentrated 50-70 fold by ultrafiltration or by lyophilization.

The protein and immunoglobulin concentrations of 15 samples of unconcentrated fluid from 15 animals were measured (table 3). The immunoglobulin content of the fluid was 0.04 mgm/ml, or 0.2% that of serum.

Cellulose acetate electrophoresis of samples of concentrated rete testis fluid revealed an $\alpha$ peak, an albumin concentration of 0.17 mgm/ml, or about 0.4% that of serum and traces of other proteins (figure 9). A higher tubule permeability to albumin than to globulin was reported previously (Mancini et al, 1965).

Ram rete testis fluid and serum were analysed immunoelectrophoretically using rabbit antisera to sheep serum and to sheep rete testis fluid (Figure 10). $\beta_2$M, gamma$_1$- and gamma$_2$-globulin and the $\beta_{1C}$ component of complement were all detected in the rete testis fluid. Two antisera to the fluid both contained antibodies reacting with both serum and rete testis fluid. The antibodies were totally absorbed out by ram serum proteins in the diffusion test of Wadsworth and Hanson (1960). G-100 Sephadex filtration of unconcentrated rete testis fluid

produced fractions identical in distribution with those of ram serum. There thus appear to be no large antigens specific to rete testis fluid. Antisera to ovine luteinising hormone and ovine follicle-stimulating hormone did not react with the fluid in gel precipitation tests.

The results described above did not differ amongst rams showing wide seasonal variations in spermatogenesis.

## DISCUSSION

The protein and immunoglobulin content of rete testis fluid is very low. The levels present in seminiferous tubule fluid may be even lower because (1) the rete testis is more permeable than the seminiferous tubules (see earlier) and (2) inflammation around the cannula may have elevated levels of serum protein. Immunoelectrophoresis and sephadex filtration reveal the presence of serum proteins only, but the cellulose acetate trace shows that the distribution of these proteins is unlike that of serum. The relatively higher level of the smaller albumin molecule is understandable, but the $\alpha$ protein peak is more puzzling. It seems to be predominantly composed of $\alpha_2$-Macroglobulin, a large molecule, the presence of which is associated with high mitotic activity (Berenblum, Burger & Knyszynski, 1968; Heim, 1968). The peak is not an artifact due to selective concentration of large molecules, for the same result was obtained with two methods of concentration but not when diluted serum was reconcentrated by either method.

The quantitative findings reported here are fully in agreement with those which failed to detect intratubular globulin qualitatively (Mancini *et al*, 1965; Waites *et al*, 1966; Raj Gupta *et al*, 1967). The blood-testis barrier acts as an immunological barrier, and explains the failure of the natural antibody to cause damage.

The barrier to acriflavine develops between day 5 and

day 20 in the rat before the appearance of the first spermatozoal autoantigen (Kormano, 1967a). The immunological barrier will therefore prevent the leakage of immunogen to immunologically responsive sites as well as restricting the entry of antibodies once induced. Traumatic injury to monkey or guinea-pig testes induced an immune response to spermatozoa which affected only the traumatized testis and not the contralateral testis with its barrier still intact (Raitsina et al, 1967).

The presence of this highly effective barrier means that for an immune reaction to spermatozoa to prove effective either the barrier must be weakened or the immune response must be of high titre or avidity.

## Chapter 3

# EVIDENCE THAT THE IMMUNOLOGICAL BARRIER IN THE TESTIS AFFECTS THE EXTENT AND PATTERN OF DEVELOPMENT OF AUTOIMMUNE ASPERMATOGENESIS IN THE GUINEA-PIG

## INTRODUCTION

The presence of an immunological barrier has been demonstrated quantitatively in the ram testis and qualitatively in the testes of rat and guinea-pig. The histological studies described in this chapter provide evidence favouring a protective role for the barrier following isoimmunization with testis. Immunological damage is shown to occur most readily at the rete testis, where the barrier is least efficient, and then to spread to the rest of the testis.

MATERIALS AND METHODS

Histology

Tissues were fixed in Susa, and 6μ sections were stained with eosin and haematoxylin. Serial sections were cut at 30μ thickness.

Classification and scoring of spermatogenic stages

Each tubule examined in sections of guinea-pig testis was classified as one of eight spermatogenic stages, the features of which are outlined in table 4. 900 tubules were counted in each testis. Sections were cut at random intervals through the testis, and each section was scanned along horizontal courses three field widths apart (x40). Every tubule section entirely within the field was scored.

Partially damaged testes were obtained from guinea-pigs isoimmunized with testis as described below.

Immunization with homologous testis

Male guinea-pigs were injected intradermally in the back of the neck at day 0 and day 12 with homologous testis (100 or 200 mgm/Kgm) homogenised with an equal volume of Freund's complete adjuvant.

Skin testing

Bellies were shaved and depilated. Injections of 0.1 ml

of the supernate of freeze-thawed guinea-pig testis homogenate were made intradermally and the injection sites scored at 2, 8, and 24 hours for erythema and induration.

Cytotoxic assay

Test and control sera were heat inactivated and serially diluted in 2 volumes of diluent (45% Medium 199:45% Hanks BSS: 5% Fetal calf serum: 5% phosphate buffer, pH7.2). To each tube were added 1 volume of complement (1/5 fresh guinea-pig serum) and 1 volume of cells ($8x10^6$/ml). The testicular cells were prepared by teasing tubules in diluent and lightly centrifuging off the debris. The ratio of round spermatids to spermatozoa was adjusted to 1:1 by the addition of epididymal spermatozoa. Tubes were incubated at 37°C for 30 minutes, and the cells scored for viability by the oesin penetration test. One hundred cells of each cell type (spermatozoa or round spermatid) were scored in each tube. Each serum was usually tested two or three times.

The assay for spermatoxicity was performed similarly, but epididymal spermatozoa only were used in the cell suspension.

Absorption of cytotoxic antisera

Cytotoxic sera were divided into 3 aliquots of 0.2 ml. One was mixed with epididymal spermatozoa (final concentration 10 or 40 x $10^6$/ml), the second with the same concentrations of round spermatids obtained from a 7 week old testis, and the third with

diluent only. The tubes were incubated with shaking at 37°C for 2 hours and the cells removed by centrifugation.

Complement fixation

One volume of guinea-pig antiserum was serially diluted in barbital complement fixation buffer (Oxoid). One volume of complement (4 M.H.D.) and one volume of guinea-pig spermatozoa ($15 \times 10^6$) were added to each tube. Tubes were incubated for 90 minutes at 37°C, following which 2 volumes of 1% sheep red blood cells with 1/800 haemolysin (4 M.H.D.) were added. Tubes were incubated for a further 30 minutes and scored for haemolysis. Controls were (1) Diluent + complement + spermatozoa (2) Antiserum diluted $\frac{1}{2}$ + diluent + spermatozoa (3) Antiserum diluted $\frac{1}{2}$ + complement + diluent (4) Normal serum + complement + spermatozoa.

Passive cutaneous anaphylaxis and immune fluorescence were performed as described in Chapter 1.

RESULTS

The patterns of testicular damage following isoimmunization of guinea-pigs with testis.

Testes of 50 mature guinea-pigs were examined between day 7 and day 70 of immunization. Three histological patterns were distinguished.

(1) No damage.

This was found in controls, which were immunized twice with adjuvant alone (5 animals) or with 100 mgm. Homogenates of guinea-pig liver, kidney, brain, spleen or muscle (one animal each). A few guinea-pigs immunized with testis also showed this pattern, despite the presence of positive immediate and delayed skin responses and of cytotoxic and anaphylactic antibodies.

(2) Extensive damage but no cellular invasion.

Excurrent ducts were devoid of spermatozoa. The contents of the seminiferous tubules were completely destroyed with the exception of the Sertoli cells, and interstitially there was fibrosis and an occasional focus of lymphocytes. This pattern occurred in all animals examined at over 30 days post immunization and in a few examined earlier, and probably represents a post-inflammatory state. All of these animals showed weak delayed skin responses, twelve out of twenty possessed anaphylactic antibodies and sixteen out of twenty possessed cytotoxic antibodies.

(3) Inflammation.

This pattern of damage was characterised by partial or complete invasion of the testis and excurrent ducts by eosinophils, mononuclear cells and neutrophils and occurred 14-30 days after the initial immunization. Frequently, only the rete testis and ductuli efferentes were invaded (figures 20-22). Involvement of the epididymis as well was less common and often appeared to occur due to spread from the rete down the excurrent ducts without interstitial inflammation (Figure 23). Large interstitial granulomata were present in other epididymides (Figure 24). Damage occurred least frequently in the body of the testis and only when also present at the rete. More invasive cells were present interstitially than within tubules (Figure 25), although cellular invasion of tubules was massive near the rete testis (Figure 26) and obvious elsewhere (Figures 27, 28). Cells evidently enter tubules directly (Figure 29) and not only by passage along the tubule from the rete testis. Intratubular eosinophils were frequently degranulated. Lymphatics were distended with mononuclear cells (Figure 30). In many testes, large regions of distended but otherwise normal seminiferous tubules indicated that the inflammation at the rete testis had blocked the outflow of rete testis fluid (Figure 31). All animals showing this pattern had immediate and delayed skin responses and all possessed anaphylactic and cytotoxic antibodies. Histological study of the 24 hour skin response of these animals revealed eosinophil, mononuclear cell and neutrophil accumulations very similar to those in the testis (Figure 19).

In the type 3 testes normal, distended and invaded tubules occurred in zones and an examination of serial sections showed that each zone represented the convolutions of one or two tubules cut repeatedly.

Ten tubules in one partially damaged testis were traced through serial sections from the rete testis into the body of the testis and scored for damage and spermatogenic stage. The results for a typical tubule are given in table 5. Stages 5-8 were damaged in the first part of the tubule, but further from the rete testis damage was absent. The selective sensitivity to damage of different spermatogenic stages which is suggested by this result was examined statistically on five partially damaged testes.

First, 900 tubules in each of four normal testes were assigned to one of the eight spermatogenic stages or to a ninth 'damaged' stage. The data was tested for homogeneity in a $X^2$ test (P=0.01) and pooled. The frequency of each stage was then compared by $X^2$ (P=0.01) with that for the same stage in each of five partially damaged testes. The results are presented in table 6. There is an increased susceptibility to damage of the tubules in the later spermatogenic stages.

The results presented here indicate:-

(1) that leucocytes are involved in the pathogenesis of autoimmune aspermatogenesis. The similarity between the skin reaction at 24 hours and the testicular inflammation strongly indicates

damage by cellular immunity. However, a humoral response may also be involved, and antigen-antibody complexes are known to attract eosinophils, macrophages and neutrophils (Litt, 1963; Cohen, Sapp, Rizzo and Kostage, 1964; Hirsch, 1965; Nelson, 1968).

(2) that there is a differential sensitivity to immune attack of various parts of the ~~testis~~ male genital tract, the rete testis and ductuli efferentes being more vulnerable than the testis or epididymis. Evidence was cited earlier that the rete testis was more permeable than other parts of the tract, and the immunological barrier may also be less effective here.

(3) that within the testis there is a differential sensitivity to immune attack of tubules at different stages of spermatogenesis. This could be due to a variation with spermatogenic stage either of the immunological barrier or of the susceptibility of germinal cells to immune attack. It is unlikely to reflect hormone deprivation of the more advanced spermatogenic stages - perhaps due to a depression of testosterone production by interstitial inflammation - for two reasons. First, the segments of damage are obviously immunological, with cellular invasion. Second, in the damaged segments all cell types are damaged not just the most advanced ones; the inflammation caused by the reaction with mature cells is presumed either directly to damage earlier cells or to increase local antibody levels by a local "leakiness" of the immunological barrier.

These three conclusions are now examined more closely.

The relationship between the occurrence of autoimmune damage and the types of immune response induced.

Immune cells are frequently cited as the sole cause of autoimmune aspermatogenesis, but the evidence for this is weak (see introduction). One method of investigating the relationship between the appearance of an immune response to spermatozoal antigen and the occurrence of damage *in vivo* is to follow the immune response in individual animals and to remove testes appropriately. A pilot experiment designed on these general lines was undertaken. The work was pre-empted by the report of Voisin *et al* (1968) who used a similar approach but did not serially test individual animals, assuming all animals to respond identically and sampling at intervals. A most important feature in the experiments of Voisin and Toullet was the identification and use of purified autoantigens. In the pilot experiment, whole testis homogenate was used on the assumption that only one autoantigen was present in the testis, and this greatly reduced its value.

Mature animals were immunized with testis (100 mgm/kgm) on days 0 and 14. Each animal was bled once every three or four days and sera were examined for cytotoxic, complement fixing and PCA antibodies. Animals were sampled at intervals between day 7 and day 30 and skin tested. Twenty four hours later they were killed and the testes examined.

Some animals showed a large antibody response 6-7 days after

the first immunization (upper graphs in figures 32, 33 and 34) and others only three days after the secondary booster (lower graphs in figures 32-34). The pattern of response in any one animal often differed for different types of antibody. This is probably due to differential stimulation by antigens S, P and T, for S and P evoke PCA antibodies, P and T evoke complement fixing antibodies and T evokes cytotoxic antibodies (Voisin et al, 1968). The animals with damaged testes are asterisked in figures 32-34, and it will be seen that no particular pattern of immune response correlates with damage.

The data for individual animals is presented in table 7. There is little obvious correlation between damage and delayed skin test result or antibody titre, a result in agreement with the data given in the previous section.

The only feature of the immune response related to damage, although not guaranteeing it, is the presence of high titres in one or more of the assays used. The same conclusion has been reached by Voisin et al (1968, 1969) who found no correlation between damage and the occurrence of any one type of immune response to any one autoantigen. These authors do claim that lesions are most common when delayed and immediate sensitivity reactions are both present, and this also claimed by Brown et al (1967). Cellular and humoral immune responses interact to produce a much larger local inflammation than either alone (Asherson, 1967) and a local inflammation could be an essential

step in the induction of autoimmune lesions. Whether this inflammation is mediated by cellular or humoral immune responses, or by a combination of both, does not seem to be important, although the threshold at which it is induced may vary for the different types of response.

Evidence ~~which~~ that confirms that antigen in the excurrent duct is less well protected than that in the seminiferous tubule.

If the contents of the rete testis are less protected by the immunological barrier than those of the seminiferous tubules, induction of autoimmune aspermatogenesis should be more difficult in testes with antigen only present in the tubules than in those with antigen present in the excurrent duct also.

This thesis was tested using maturing male guinea-pigs. Animals were divided into four groups by age (34, 44, 54 and 64 days). One animal in each group was injected with Freund's complete adjuvant alone. Of the remainder, half were injected twice with 100 mgm/kgm and half twice with 200 mgm/kgm of homologous testis in complete adjuvant. A fifth group of mature guinea-pigs were injected with 100 mgm/kgm only. This ensured that at least half of the guinea-pigs in the immature groups received a total testis injection which equalled or exceeded that given to mature animals. The same testis homogenate was used throughout. At day 19 the animals were skin tested, and at day 20 they were killed. Their sera were tested for a $\gamma_1$-globulin response by PCA (Ovary, Benacerraf & Bloch, 1963) and for a

$\gamma_2$-globulin response by spermatoxicity (Bloch et al, 1963). Testes from injected animals were compared for maturity and for damage with those from adjuvant controls. The presence of antigen in the testes and of autoantibody in the serum capable of reacting with this antigen were tested by immune fluorescence.

The results are summarised in table 8. Figures 35, 36 and 37 show sections of a testis from an animal in group 3 after treatment with (a) autologous serum diluted $\frac{1}{10}$ (b) control normal serum diluted $\frac{1}{10}$ and (c) saline, each followed by fluorescein-conjugated antiserum to $\gamma$-globulin.

The immune response does not vary significantly in the different groups and this indicates that the ability to respond immunologically to autoantigens is fully developed within 34 days of birth in the guinea-pig. Autoantigen was present in the seminiferous tubules of all animals. However, testicular damage occurred in twentyfour of the twentynine animals (89%) with antigen in excurrent ducts but in only two of the twentyfour animals (8%) without substantial antigen in the excurrent ducts. In the two exceptions damage was restricted to the rete testis which contained sloughed spermatocytes and early round spermatids. Over sixty animals of group (3) type have since been examined and only about four of these showed damage, which was always confined to the rete testis.

The result is fully supported by the experiments of Bishop, Narbaitz & Lessof (1961) who used a similar system for rather

different purposes. Examination of their data reveals that no guinea-pig immunized neonatally with homologous testis became aspermatogenic until about 80 days after birth despite the presence of a detectable immune response. At this time, substantial spermatozoal antigen first leaves the seminiferous tubules.

The result emphasises the efficiency of the immunological barrier of the seminiferous tubule relative to that at the rete testis and this efficienty parallels that of the blood-testis barrier at exclusion of albumin and dyes (Waksman, 1960). It seems moreover that at the immunizing doses used here, damage to the rete testis is a necessary prerequisite for testicular damage to occur.

Evidence that explains why the later spermatogenic stages are more readily damaged than the earlier stages.

Two possible explanations for the greater sensitivity of the later spermatogenic stages were proposed earlier. There is little evidence supporting a reduction in the effectiveness of the tubular barrier during the later stages of spermatogenesis. Suvanto & Kormano (1968) have claimed differences in tubule motility at different stages, and there is firm data on changes in the Sertoli cell ultrastructure with progression of spermatogenesis (Roosen-Runge, 1962). This evidence does not bear directly on the present problem, which could be most convincingly solved by micropuncture

studies.

An increasing sensitivity to immune attack of the cells at the later stages would be expected for if spermatozoa were more autoantigenic than their precursor cells then the chance of an antibody-antigen reaction and hence of local damage might be greater. The synthesis of potentially antigenic non-nuclear protein continues up to the elongated spermatid (Monesi, 1965), and spermatozoa are known to be more immunogenic that their precursor cells (Katsh, 1960). The cytotoxicity of serum is dependant upon the concentration of antigens on the cell surface (Moller & Moller, 1962), for two adjacent antibody molecules are apparently necessary to fix complement and cause lysis, and the chance of this occurring is less with fewer antibody binding sites. If spermatids possess less surface antigen than spermatozoa then when equal numbers of each cell type are exposed to suitable cytotoxic antibody and complement concentrations, the spermatozoa should be more readily lysed. Also the absorptive capacity of spermatozoa for cytotoxic sera should be greater than that of spermatids. These two predictions have been tested experimentally.

Each of fifteen guinea-pig antisera were examined at least twice for their relative cytotoxicity for homologous spermatozoa and round spermatids. Typical results for three sera are presented in table 9. The difference in relative cytotoxicity was similar in all fifteen sera. However, the absolute cytotoxicity of the various sera differed and the crude data could

not be simply combined. Thus, if the values in table 9 are plotted graphically, the pairs of curves resulting are of similar shape but displaced laterally with respect to each other along the horizontal axis. Antiserum (1) is approximately half as cytotoxic as antiserum (2) for both spermatozoa and spermatids, and presumably contains half the quantity of specific antibody. By moving all the values of (2) for both the spermatozoa and spermatids one place to the left, the absolute strengths are made to appear more or less similar. Effectively, the curves are being shifted along the horizontal axis. If this is similarly done for all fifteen antisera the mean values for percentage cells dead at each 'dilution step' can be calculated and plotted as shown in figure 38. A more legitimate mathematical comparison can be made by plotting the two cytotoxicity curves for each antiserum and measuring the difference in value of the lethal dose 70 (LD70) of the antiserum for spermatids and spermatozoa. This procedure eliminates differences in absolute cytotoxicity of sera and gives a mean difference in LD70 of 1.83 $\pm$ 0.02 dilution steps ($\pm$S.E.)

The data on individual and combined antisera clearly demonstrate that the cytotoxicity of the antisera for spermatozoa is greater than that for spermatids. The highly resistant group of spermatids (10-15%) were round, possessed a very small idiosomal vesicle and were presumed to be early spermatids.

Equal volumes of spermatozoa and spermatids were used to

absorb eight of the antisera. Results for three typical sera are given in table 10. The absorptive capacity of spermatozoa is greater than that of spermatids.

Cytotoxic autoantibodies are produced to autoantigen T (Voisin et al, 1968), and the evidence presented here indicates that the concentration of this antigen on the cell surface increases as spermatogenesis proceeds. The increase in surface autoantigen T during spermatogenesis is no doubt paralleled by the other three autoantigens S, P and Z (Voisin et al, 1968). The spermatogenic cells will therefore become increasingly sensitive to attack not only by cytotoxic but also by any other humoral or cellular immune response.

## DISCUSSION

The evidence produced in this chapter taken with that of the other authors suggests that the following sequence of events may occur during the pathogenesis of autoimmune damage to the testis.

First sufficiently large quantities of spermatozoal antigen(s) must reach immunologically competent cells in a state which induces a response. The dose and method of presentation of the antigens (s) will affect the magnitude, sequence and balance of the various types of immune response.

If the level of a suitable immune response rises sufficiently it will overcome the protective effect of the testis barrier at its weakest point, the rete testis, initiating an immune reaction with spermatozoal antigens. This may cause a booster immunisation due to systemic release of more antigen.

The effect of this reaction may depend on the type of immune response involved. If the antibody-antigen interactions do not result in a local inflammation, further damage may not occur or may be restricted to distension of seminiferous tubules due to blockage at the rete testis. However, $\gamma_1$ and $\gamma_2$ antibodies and immune cells are all known to produce local inflammation in the presence of antigen in some circumstances, and a combined cellular and humoral attack is much more potent than either alone (Asherson, 1967). The type of immune response may not therefore be important, although some may be more effective than

others.

The local inflammation will spread with the flow of fluid into the epididymis and may spread back through the seminiferous tubules. The inflammation in the interstitial tissue around the rete testis will also affect adjacent tubules. By these two processes the protective effect of the immunological barrier in the seminiferous tubule could be reduced allowing leakage into the tubules of antibody. The process would then tend to be auto-catalytic, spreading out from the rete testis by tubular and interstitial paths.

During the early stages of inflammation the antibody and complement levels within the tubules will be very low, and may therefore only damage the more antigenic cells.

When the antibody-antigen interaction ceases or is reduced, the inflammation will similarly cease and the integrity of the barrier will be restored. This may occur without complete destruction of the testis, especially when the immune response is weak or not of the optimal type.

This proposed pathogenesis of autoimmune aspermatogenesis is open to experimental testing. In the following chapter evidence is presented demonstrating that tubular permeability does in fact rise during the inflammatory phase of damage. In chapter 5, experiments are described in which the tubular barrier of the immature testis is weakened, thus increasing the incidence of autoimmune lesions.

Chapter 4

# DETECTION OF A CHANGE IN THE PERMEABILITY OF THE IMMUNOLOGICAL BARRIER OF THE GUINEA-PIG TESTIS FOLLOWING ISOIMMUNIZATION WITH TESTIS.

## INTRODUCTION

Two ways of detecting changes in the barrier permeability following isoimmunization with testis are theoretically available. The more quantitative approach is to cannulate the rete testis and analyse changes in the rete testis fluid. This was attempted unsuccessfully in rams, when no autoimmune response to spermatozoa could be detected (unpublished work with B.P. Setchell). The method is however liable to error, for the cannula inserted in the rete testis might profoundly modify the course of the pathogenesis.

A qualitative result can be obtained by measuring changes in the distribution within the testis of a marker substance such as acriflavine. The failure of intravenously injected dyes to stain tissues is not necessarily due to the presence of barriers. Convincing controls are therefore needed before a change in staining pattern can be equated with a change in the efficiency of barriers. Three experiments are described in this chapter. The first two establish the validity of applying a tracer technique to the third, which investigates changes in the testicular permeability to acriflavine following isoimmunization with testis.

MATERIALS AND METHODS

Acriflavine injections.

Acriflavine (20 mgm/kgm) was injected subcutaneously into rats or guinea-pigs five hours prior to sacrifice. Testes were removed and small blocks snap-frozen in Arcton precooled by liquid nitrogen. The blocks were freeze-dried, vacuum embedded and sections were cut and mounted in xylol for viewing on the Zeiss Photomicroscope by dark-field fluorescence (HBOW Mercury Vapour lamp, primary filter 1, secondary filter 47). A 0.02% solution of acriflavine was injected intratesticularly in one animal as a positive control to show that the tubular contents could stain (figure 39). The criterion for positive staining was the presence under dark field illumination of clearly identifiable fluorescent nuclei, with distinct chromatin strands or nucleoli. Hazy cytoplasmic fluorescence, vivid yellow or green blobs and fluorescent debris, often due to necrotic nuclei were not scored as positive. Where two levels of fluorescence brighteness were distinguishable in one section, the brighter was graded as ++ and the dimmer as +.

Cadmium chloride injections

A 2% cadmium chloride solution was injected subcutaneously into twenty guinea-pigs at a dose of 20 mgm/kgm. Sample animals were killed and the testes examined for damage and acriflavine

permeability 5, 12, 24, 36 and 48 hours later. Negative controls were (a) on animals injected 36 hours prior to sacrifice with $CdCl_2$ only, and (b) five animals injected 5 hours prior to sacrifice with acriflavine only.

Suppression of gonadotrophins in neonatal rats.

Sixty-three newborn male rats were divided into four groups. Group 1 (18 animals) were given daily subcutaneous injections of arachis oil; Group 2 (14 animals) were given oestradiol benzoate in arachis oil daily, 10 µgms day 1-10, 20 µgms day 10-20, 30 µgms day 20-40, 40 µgm day 40-60; Group 3 (17 animals) were given oestradiol benzoate in orachis oil daily, 30 µgms day 1-10, 40 µgm day 10-20, 50 µgm day 20-40; Group 4 (14 animals) were given oestradiol benzoate in arachis oil daily, 50 µgms day 1-10, 70 µgms day 10-20, 90 µgms day 20-40. Animals from each group were sampled at day 3, day 15, day 25 and day 50, and the testes examined histologically and for acriflavine distribution. The maximum equatorial diameters of the testes in each group were also compared, and the alkaline phosphatase staining examined.

Hypophysectomised mature rats.

Forty hypophysectomised and thirty-two sham operated Sprague-Dawley male rats (70 days old) were supplied by Carworth-Europa. They were divided into five groups of 10 or 5 operated and 8 or

4 control animals. Groups were sacrificed at day 2, 5, 10, 25 or 35 days after operation, and the testes were examined histologically and for acriflavine distribution. Completeness of hypophysectomy was examined by inspection of the sella turcica at sacrifice, and by comparison of body and testis weights of sham operated animals with those of operated animals.

Alkaline Phosphatase Staining

Thin slices of testis were fixed, cleared and embedded via 90% alcohol, 96% alcohol, absolute alcohol and benzene. 10μ sections were cut, and mounted on slides without floating on water. Sections were then incubated at 37°C for 5, 10, 15 or 30 minutes in the following solution,

| | |
|---|---|
| 2% Sodium glycerophosphate | 20 mls |
| 1% Calcium chloride | 20 mls |
| 2% Sodium veronal | 20 mls |
| 0.1% Magnesium sulphate | 2 mls |
| Distilled water | 38 mls |

Control sections were incubated in a medium containing no sodium glycerophosphate. The alkaline phosphatase releases phosphate from the substrate. A cobalt phosphate precipitate is formed by incubation with cobalt nitrate, and then a black cobalt sulphide precipitate forms on incubation with ammonium sulphide. Sites of black staining indicate the presence of

alkaline phosphatase.

Skin testing, immune fluorescence and immunization of guinea-pigs were as described previously.

RESULTS

The effect of the pituitary on the blood-testis barrier of the rat.

The blood-testis barrier appears at the same time as the descent of the testes, the changes in blood flow that lower testicular temperature, the initiation of testis fluid secretion and the appearance of cadmium sensitivity which reflects changes in capillary walls (Parkes, 1966; Kormano, 1967a; Setchell, 1968; Clegg, Niemi & Carr, 1969). The secretion of pituitary gonadotrophins and therefore testosterone is also rising at this time and stimulates the descent of the testis (see Parkes, 1966). The gonadotrophins could also be responsible for the barrier changes and the initiation of testis fluid secretions. However, other barriers appear at the same time in the brain and gastrointestinal tract, and the appearance of all these barriers is associated with a rise in alkaline phosphatase staining (Stern & Peyrot, 1927).

There is some evidence that hypophysectomy or variation in gonadotrophin secretion does not affect the blood-testis barrier of the mature animal. Thus the seasonal variations in the output of ram spermatozoa were not paralleled by corresponding changes in rete testis fluid production or composition (Johnson & Setchell, 1968; Setchell, Dawson & White, 1968). Also, hypophysectomy of mature rats did not eliminate the rubidium rejecting compartment from the testis (Setchell et al, 1969).

Total secretion of testis fluid fell 21 days after hypophysectomy in the rat, but the volume of fluid produced was similar to that from a normal scrotal testis of the same size (Setchell, 1968). Acute injections of oxytocin into a ram with a cannulated rete testis did not affect flow or composition of rete testis fluid (Setchell & Linzell, 1968). In contrast to these results is the finding that alkaline phosphatase staining in the testis disappears within 26 days of hypophysectomy, but is restored by pituitary extract (Dempsey, Greep & Deane, 1949).

The role of the pituitary in the development and maintenance of the blood-testis barrier has been investigated using the distribution of acriflavine as a marker.

(1) Development.

Daily administration of oestrogens to neonatal rats depresses gonadotrophin and thus testerone production. Oestrogens were administered to group 2 of the neonatal rats at a dosage known to prevent testis descent and spermatogenesis (Steinberger & Duckett, 1967). Groups 3 and 4 were treated with doses 3 and 6 times greater. The testes were examined at 3, 15, 25 and 50 days and compared with control testes for (a) staining pattern with acriflavine (indication of barrier development) (b) presence of tubular lumen (indication of fluid production) (c) state of spermatogenesis (d) testicular size (3) abdominal or scrotal position of testes (f) alkaline phosphatase staining of testes.

The results are tabulated in table 11.

(a) The contents of the three day rat testis stained vividly with acriflavine (figure 40). Treatment with oestrogens failed to prevent the development of the tubular barrier between days 3 and 15, even though interstitial tissue fluoresced (figures 41 and 42).

(b) A tubular lumen was absent in both control and oestrogen-treated 3 day and 15 day animals, but was present in the older animals from all four groups (figures 43, 44, 45).

(c) Spermatogenesis was restricted to the same stage regardless of the oestrogen dose used (figures 44 & 45). This was also the stage reached by testis tissue cultured in vitro in the absence or presence of gonadotrophins and testosterone (Steinberger & Steinberger, 1967).

(d) Testicular size reflects not only the state of spermatogenesis but also the lengthening of the tubules which occurs following gonadotrophin secretion. Oestrogens depressed testicular growth, and higher oestrogen doses were more effective than lower doses.

(e) All testes in oestrogen treated animals were abdominal.

(f) Alkaline phosphatase was present in the walls of the capillaries and seminiferous tubules in the mature rat (figure 54). In the 3 day rat, the enzyme was absent (figure 46), but had appeared by day fifteen in the control animals of group 1 (figure 48). In oestrogen treated animals, however, alkaline phosphatase staining was only vivid in the capillaries. The walls

of the seminiferous tubules stained palely and patchily (figures 48 and 49). By day 25 both the vascular and tubular distribution and intensity of staining were similar for all groups. In the oestrogen treated animals, the elongated peritubular cells of layer 2 were clearly distinguishable as individual stained cells.

(2) Maintenance.

The presence of the blood-testis barrier in hypophysectomized rats would demonstrate the independance of the barrier maintenance of any pituitary hormone including the gonadotrophins, prolactin and oxytocin. Animals were divided into groups as described in materials and methods. Of the hypophysectomized animals in each group, one was given a saline injection and the rest were given acriflavine.

The acriflavine staining patterns are summarized in table 12. Figures 50, 51 and 52 show the lack of change in acriflavine permeability 35 days after hypophysectomy, despite spermatogenic failure which was detected within 10 days of operation (figure 53). Cellular debris within the tubules fluoresces a dull yellow and is quite distinct from the green nuclear staining of acriflavine.

Alkaline phosphatase staining is undiminished 35 days after hypophysectomy (figures 54, 55 and 56).

Functional or surgical hypophysectomy suppressed spermatogenesis, caused testicular atrophy, prevented testicular descent

and reduced body weight. No effect on the blood-testis barrier was detectable. A slight retardation in the appearance of alkaline phosphatase was observed, and this enzyme is frequently associated with active secretory epithelia. However, removal of the adult pituitary did not affect distribution of the enzyme and there is thus no good evidence for a pituitary effect on testis fluid secretion. Dempsey et al (1949) could detect no testicular alkaline phosphatase 26 days post operatively; unless the technique used here is more sensitive, it is difficult to explain this discrepancy.

The development and maintenance of the blood-testis barrier are therefore either independant of the pituitary or very sensitive to its secretions, and this conclusion is in agreement with that of other workers (see earlier).

The effect of cadmium chloride on the blood-testis barrier of the guinea-pig.

Cadmium exerts a selective toxic effect on the blood vessels of the testis and caput epididymidis (Gunn, Gould & Anderson, 1963; Chiquoine, 1964; Mason & Young, 1967) and investigations into the changes in blood flow and vascular permeability suggest that the primary site of action is the end-arterial bed (Niemi & Kormano, 1965; Waites et al, 1966; Clegg & Carr, 1966; Raj Gupta et al, 1967; Kormano, 1968). Raj Gupta et al (1967) showed that not only did serum proteins leave the blood vessels

after administration of cadmium, but that 12-24 hours later they were also detectable inside seminiferous tubules. This strongly suggests a breakdown of both barrier components and the acriflavine distribution following cadmium administration was used to investigate this.

The animals were treated and the testes examined as described in materials and methods. The results are summarised in table 13. Staining of interstitial nuclei became stronger within twelve hours of cadmium administration suggesting increased vascular permeability (figures 57 and 58). After 24 hours there was a breakdown of the tubule barrier which was initially focal corresponding to similar foci seen histologically in figure 59, but by 48 hours the whole testis was involved (figures 60 and 61). Examination of the freeze-dried sections by switching from phase to fluorescence revealed a complete oorrelation between damaged tubules or parts of tubules and intratubular fluorescence. Some apparently undamaged tubules also stained and this suggests that breakdown of the barrier occurred early in the production of damage. Other normal tubules in partially damaged testes were not stained, and neither were tubules damaged by cadmium but present in the testes of animals not given acriflavine (negative control, figure 62).

Damage within the tubules has been regarded as due to a direct effect of ischaemia on spermatogenesis. However, experimental ischaemia of the perfused testis causes a cessation in

the flow of the rete testis fluid within two minutes (Linzell and Setchell, 1968), and the production of this fluid is probably closely linked with the selective exclusion by the blood-testis barrier of serum components. This suggests a new explanation for the damage caused by cadmium. The tubule barrier could be the site primarily affected by the ischaemia following cadmium administration, and after the breakdown of this barrier the influx of serum components might secondarily initiate the damage to tubule contents. This secondary damage could be due to non-specific changes in the micro-environment of the cells, to cadmium interference with zinc metabolism in the tubule (Parizek, 1960) or perhaps due to the cytotoxic action of the natural antibody on the germinal cells.

Acriflavine clearly provides a sensitive test for the integrity of the testis barrier, and its changes in distribution are similar to those reported for gamma-globulin and albumin (Raj Gupta et al, 1967). The dye was therefore used to test for permeability changes in testes damaged by isoimmunization with testis.

Changes in the blood-testis barrier following immunization of guinea-pigs with homologous testis.

In chapter 2 descriptions were given of three patterns of histological damage following isoimmunization in the testis.

The distribution of acriflavine within the testis was studied for each of these patterns.

The undamaged testes of all control and a few test animals fluoresced weakly in blood vessels and interstitially but not intratubularly (figure 57), and thus did not differ from normal.

Testes of damaged but non-inflamed testes showed strong vascular and interstitial staining. No intratubular nuclei stained even when only a few microns from those staining in the tubular membrane (figures 63 and 64).

In the inflamed testes faint but clear intratubular staining was regularly observed in regions of cellular invasion (figure 65). The fluorescence could readily be distinguished from the auto fluorescent granules of dead cells, but was much fainter than that seen after cadmium damage. This may reflect incomplete breakdown of the barrier. The permeability rise is clearly associated with the invasive phase of damage and may be related to eosinophil degranulation or the release of acrosomal enzymes. The leakiness of the barrier is shortlived, for it is not present in the damaged but uninflamed testes described above.

The presence of gamma-globulin in the inflamed tubules was demonstrated by staining sections directly with fluorescein-conjugated anti gamma-globulin (figure 66). Tubules at a similar stage of spermatogenesis in non-inflammed testes failed to stain (figure 67; see also figure 37), despite the presence of circulating antibodies to spermatozoa.

DISCUSSION

The failure of acriflavine to stain the normal testis could be due to factors other than the presence of a permeability barrier but this is unlikely. The lack of staining is not due to an inability of normal tubule cells to take up the dye as shown by the positive control in figure 40. The dye can reach the testis, for vascular and interstitial cells stain normally and so do tubular cells following cadmium administration when the blood flow is greatly reduced (Waites et al, 1966). Binding of the dye by cells outside the tubule could prevent tubular staining. If this was so, the total binding capacity would have to be very great, for the seminiferous tubules of guinea-pigs injected subcutaneously or intravenously with doses of acriflavine as large as 200 mgm/kgm up to ten hours previously still failed to stain. This evidence plus the similarity between the acriflavine distribution in the testis and that of many other substances justify the assumption that a barrier prevents the tubule contents in a normal testis from staining.

Acriflavine staining within a pathological tubule is not due to a greater permeability of dead cells to the dye, for dead and dying nuclei in the tubules of hypophysectomised animals did not stain. However, following $CdCl_2$ administration, nuclei did stain and there is good independant evidence suggesting that the barrier has become less effective in this condition

The distribution of acriflavine under various circumstances

seems to parallel that of serum protein qualitatively. Thus, both are partially restricted by the vascular barrier and totally by the tubular barrier. These barriers break down in the same sequence following cadmium administration for both acriflavine and serum proteins (Raj Gupta et al, 1967). Following isoimmunization with testis, gamma-globulin was only detectable by direct immune fluorescence in those tubules also permeable to acriflavine and invaded by cells. Uninvaded tubules in the same testis contained neither gamma-globulin nor acriflavine at levels detectable by techniques used here. Acriflavine and serum protein distribution are also similar in the female genital tract, for both can be readily detected in follicular fluid whereas neither enter uterine secretions to any great extent (see later).

Voisin et al (1969) recently reported that the uptake of Evans' blue dye by the testis rose during development of auto-immune aspermatogenesis, and suggested that this was due to increased vascular permeability. The results presented here localise the permeability changes more precisely and show that immunoglobulins also leak in.

The data fully supports the pathogenesis of autoimmune aspermatogenesis proposed in the previous chapter, and confirms a critical role for the inflammation generated at the rete testis. In the absence spermatozoal antigen in the rete testis, it should be possible to induce testicular damage either by

increasing the level of immune response to overcome the tubular barrier or by weakening the tubular barrier itself. Immature male guinea-pigs were used to test these possibilities.

## Chapter 5

# THE EFFECT OF WEAKENING THE TESTIS BARRIER OR OF INCREASING THE IMMUNE RESPONSE ON THE INDUCTION OF AUTOIMMUNE ASPERMATOGENESIS.

INTRODUCTION

The level of the immune response may be increased by simply raising the immunizing dose. The blood-testis barrier is weakened by administration of cadmium chloride by traumatic lesions, and might also be weakened by nonspecific inflammations in the testis such as a local hypersensitivity response to tubercle bacilli. The testes of three groups of immature guinea-pigs (74 days) were therefore damaged by these methods twenty days after isoimmunization with testis.

INTRODUCTION

The level of the immune response may be increased by simply raising the immunizing dose. The blood-testis barrier is weakened by administration of cadmium chloride by traumatic lesions, and might also be weakened by nonspecific inflammations in the testis such as a local hypersensitivity response to tubercle bacilli. The testes of three groups of immature guinea-pigs (74 days) were therefore damaged by these methods twenty days after isoimmunization with testis.

MATERIALS AND METHODS

Immunization of guinea-pigs

This was as described previously. Animals were sacrificed at day 20.

Traumatic damage to testes

The animal was anaesthetized 24 hours prior to sacrifice and one testis exposed by a scrotal incision. A sterile mounted needle was thrust four or five times through the tunicae vaginalis and albuginea into the testis. The testis was then replaced.

Cadmium chloride injection

This was performed on the eighteenth or nineteenth day after immunization as described in chapter 3. Three groups of ten animals each were used. The dosage of $CdCl_2$ for group (1) was 6 mgm/kgm., for group (2) 7.5 mgm/kgm and for group (3) 10 mgm/kgm. Half of the animals in each group were injected 48 hours prior to sacrifice, and the remaining half 24 hours later. Prior to the injection the right testis was removed as a control. The left testis was removed at sacrifice.

Adjuvant injection

Animals were injected with testis in Freund's complete adjuvant or with adjuvant alone as described in chapter 2. At day 19, one testis was injected with 0.05 ml of Freund's

complete adjuvant, and the animal was sacrificed one day later.

Passive cutaneous anaphylaxis, spermotoxicity, skin testing and histology were carried out as described previously.

RESULTS

The effect of increasing the immunizing dose of homologous testis on the incidence of autoimmune aspermatogenesis in immature animals

Duncan-Hartley male guinea-pigs aged 54 days were divided into four groups, and each group was immunized with aliquots of the same testis homogenate emulsified with Freund's complete adjuvant. The immunizing doses were group (1) 200 mgm/kgm (8 animals), (2) 400 mgm/kgm (10 animals), (3) 800 mgm/kgm (11 animals) and (4) 1600 mgm/kgm (9 animals). At sacrifice on day 20, sera were collected and the $\gamma_1$- and $\gamma_2$-globulin responses were estimated as described in chapter 2. Testes were examined for damage. The results are summarized in table 14.

The incidence of damage does not rise with the increase in immunizing dose, although the immune response apparently does. The 3 testes with some signs of damage were only affected at the rete testis which contained spermatids.

The effect of weakening the blood-testis barrier on the induction of autoimmune aspermatogenesis in the immature guinea-pig

(1) Cadmium chloride administration.

In chapter 3 it was shown that cadmium chloride (10 mgm/kgm) destroyed the blood-testis barrier, and at lower doses cadmium may weaken it. In the experiment reported here, 74 day old guinea-pigs, isoimmunized 20 days previously with testis, were

injected with cadmium chloride (6, 7.5 or 10 mgm/kgm) 24 or 48 hours prior to sacrifice. The right testis from each animal was removed prior to the injection and was later compared histologically with the left remaining testis for signs of autoimmune aspermatogenesis. Testes from nonimmunized animals injected with $CdCl_2$ were also examined as controls. The results are summarized in table 15.

Cadmium at doses of 6 or 7.5 mgm/kgm produced ischaemic damage infrequently compared with a 10 mgm/kgm dose, which consistently produced local or extensive ischaemic necrosis of the testis. Autoimmune damage only occurred in 3 of 30 right testes removed prior to cadmium administration from animals isoimmunized with testis. In the remaining left testes autoimmune lesions were present at all sites of obvious ischaemic necrosis, but not at other sites and not in testes undamaged by $CdCl_2$. In the early stages of ischaemic necrosis macrophages and eosinophils were present outside the tubules which were then invaded at the point of weakness (figure 68). These lesions thus resemble the invasive phase of autoimmune aspermatogenesis (figure 25-28) but are very different from those of ischaemic necrosis in animals unimmunized with testis (figure 59). In testes where the lesions were still focal, the rete testis was not inflamed, and these are the only testes thus far observed with this pattern of damage.

(2) Traumatic damage.

Fourteen 54 day old male guinea-pigs were immunised with homologous testis (200 mgm/kgm) and nineteen days later the left testes were damaged by repeated puncture with a sterile mounted needle, as described in materials and methods. The left testes of ten animals injected with adjuvant only were damaged similarly as controls. The right testes were undamaged normal controls.

The results may be summarized as follows. For the ten damaged testes from nonimmunized control animals, lesions were characteristically necrotic sometimes with haemorrhage and an accumulation of neutrophils (figure 69). The contents of tubules at the site of the lesion stained with acriflavine showing that the traumatic damage had destroyed the barrier. In the 14 undamaged right testes from the experimental immunized animals, no autoimmune lesions were present. In eleven of the damaged left testes from these animals, macrophage, eosinophil and neutrophil accumulations were present at sites of the lesion (figure 70). The remaining three testes were indistinguishable from the control testes, with neutrophil accumulations only. In the eleven testes showing cellular invasion there was no damage or inflammation at the rete testis.

The immunized animals were skin tested 24 hours prior to sacrifice, and the pattern of cellular invasion in the skin was similar to that at the traumatic lesions.

(3) Testicular inflammation

The injection of Freund's incomplete adjuvant into the testes of ten animals immunized with complete adjuvant caused little inflammation. Intertubular 'spaces' surrounded by a few neutrophils were the only signs of the adjuvant deposit. The injection of complete adjuvant into the testes of ten animals immunized with complete adjuvant produced a very different picture. Within 24 hours tubules were surrounded by a mass of mononuclear cells, neutrophils and a few eosinophils (figure 71). Spermatogenesis was unimpaired. The contents of the tubules in the inflamed region stained weakly but clearly with acriflavine. The cellular invasion was presumed to be a response to the tubercle bacilli in the complete adjuvant.

Injection of complete adjuvant into the testes of sixteen animals previously immunized with testis in complete adjuvant produced a similar interstitial inflammation at 24 hours. In these testes, however, tubular invasion by cells had also occurred and spermatogenesis was impaired locally (figure 72). The acriflavine permeability of these tubules was also slightly raised, for the nuclear contents stained weakly.

Four animals immunized with complete adjuvant only were examined later than 24 hours after the intratesticular injection of adjuvant. The two examined at 50 hours were similar to those at 24 hours, apart from a reduction in the number of intertubular

neutrophils. However, by 78 hours erosion of the tubular basement membrane was occurring, mononuclear cells and neutrophils had entered the tubules and spermatogenesis was disrupted locally. (figure 73). These testes resembled those from animals immunized with isologous testis 24 hours after the intratesticular injection.

DISCUSSION

Testes with spermatozoa in the excurrent ducts are damaged by immunizing doses of 100 mgm/kgm, but a dose 16 x this fails to regularly damage less mature testes. This result conflicts with that of Katsh (1960) who used similar immunizing doses and described extensive aspermatogenesis, which was not accompanied by cellular invasion and was very similar to the areas of hypospermatogenesis seen in normal maturing testes. Katsh examined testes 30 or 60 days after immunization, and development of damage could take longer in less mature animals. The conflict between these results would not then be serious, for the additional time needed to induce damage in the immature animals could reflect the greater resistance to attack of the testis without antigen in the excurrent duct.

The effectiveness of the immunological barrier in resisting the entry of cells or antibodies must therefore be very high. If however the barrier is weakened damage results. Cadmium, trauma and local interstitial inflammation all increase the permeability to acriflavine, and presumably also allow antibodies and immune cells directed against spermatozoa to leak in from the blood and damage the tubule contents. In the absence of specific immune cells and antibodies immunological damage to the tubule contents does not occur. Two experiments by other workers give further support to those reported here. Boughton & Spector (1963) reported that lesions in one guinea-pig testis

induced an immune response to spermatozoa which did not damage the contralateral testis unless an injection of Freund's complete adjuvant was made in the contralateral footpad. Presumably, a mild general inflammation resulted from the footpad injection and weakened the blood-testis barrier sufficient to make the immune response effective. Voisin & Toullet (1969) have recently reported that animals with a humoral but not a cellular reaction to spermatozoa only show testicular damage when a general delayed-hypersensitivity to a second antigen is induced. A similar explanation could apply.

The leucocytic invasion of tubules and the impaired spermatogenesis observed three days after the injection of tubercle bacilli into the testes of animals immunized with complete adjuvant only, could have one of three causes. The natural antibody levels within the tubules could have slowly built up initiating a reaction against spermatozoa. Secondly, the testicular inflammation could have induced an immune reaction to spermatozoa which was then able to leak in. Systemic delayed-hypersensitivity responses have been elicited within 5 days (Boquet & Bretney, 1934), local responses may occur more rapidly. Injection of adjuvant into the testis or traumatizing of the testis both elicit an immune response to spermatozoa (Boughton et al, 1963); Bratanov, Dikou & Popova, 1964; Raitsina et al, 1967), the presence of which was not tested in the animals of this experiment. Thirdly, the damage could have been nonspecific, resulting from a local temperature

rise or lack of metabolites. This latter possibility of non-specific aspermatogenesis resulting from interstitial inflammation does not imply that autoimmune aspermatogenic damage itself is nonspecific. A specific immune reaction is still required both to initiate the inflammation at the rete testis and to produce damage inside the 'leaky' tubules rapidly and autocatalytically.

Two observations described in this chapter are especially important. First the rete testis is the weakest point in the normal testis and damage spreads from that point. In the results presented in this chapter, the rete testis was often quite uninflamed despite intense cellular invasions at locally induced points of weakness, and had in fact been supplanted as the initiator of testicular damage. Secondly, for the two days after the intratesticular injection of adjuvant into animals immunized with adjuvant only, the tubular membrane effectively excluded the mass of extratubular cells. The barrier also excludes most of the cells in an animal immunized actively with testis in adjuvant (figure 25). In both these conditions there is faint but clear acriflavine staining of the tubule contents. The resistance of the barrier, although weakened is clearly not totally destroyed.

The results reported in this chapter support the pathogenesis of autoimmune aspermatogenesis proposed in chapter 4.

If this pathogenesis is correct, then hope of an effective contraceptive vaccine for the male is slender. The testicular

inflammation necessary for aspermatogenesis would prove unacceptable. The induction of a milder humoral antibody, which did not elicit an inflammatory response but did agglutinate ejaculated spermatozoa, would present the danger of a violent orchitis whenever the blood-testis barrier was weakened, for example in general pyrexia. The properties and effects of one such 'soft' antibody are described in chapter 5.

## Chapter 6

# AN IMMUNOLOGICAL BARRIER IN THE ADNEXAL GLANDS OF THE MALE GENITAL TRACT OF ANIMALS AND MAN.

INTRODUCTION

In the last three chapters evidence has been produced for the presence of a powerful immunological barrier in the testis and its excurrent ducts. Spermatozoa leave the male tract at ejaculation and are transferred to the vagina or cervix in a fluid mainly derived from the male accessory glands. Several authors have produced clinical evidence that an immune response to autologous spermatozoa can affect the fertility of the ejaculate without affecting the production of spermatozoa in the testis (Wilson, 1954; Rumke, 1954; Rumke & Hellinga, 1959; Rao, Sadri & Sheth, 1961; Segal, Tyler, Rao, Rumke & Nakabayashi, 1961; Phadke et al, 1964; Fjallbrandt, 1967, 1968a). The immune response operates via the accessory gland fluids, for the spermatozoa are apparently normal in the fresh ejaculate, but agglutinate or lyse within minutes. The origin of the agglutinating and spermotoxic action has been further narrowed down to the prostatic fluid by analysis of split ejaculates (Rumke, 1969). Men producing ejaculates which agglutinate or lyse spermatozoa also possess serum agglutinins and spermotoxins with similar properties, but usually of greater potency. The immunological barrier in the testis and its excurrent duct evidently protects the spermatozoa of these infertile men more effectively than any barrier existing between the blood and the prostatic secretions. If this is so, it should be possible to demonstrate:-

(1) that the agglutinating/spermotoxic activity in both serum and seminal plasma is an antibody. This has not been rigorously tested (Rumke, 1969) and nonspecific agglutination or lysis by viruses, bacteria, mycoplasma and steroid binding hormones is well documented (Rosenthal, 1931; Wilson, 1956, Peleg and Ianconesu, 1966; Taylor-Robinson and Manchee, 1967; Boettcher, 1967; Bell, 1968).

(2) that antibodies are more readily transported into seminal plasma than into fluids of the seminiferous tubule and testicular excurrent duct. Most authors have failed to demonstrate convincingly the presence of serum immunoglobulins in the seminal plasma of various species (Grey & Huggins, 1942; Hermann, Licht, Kentel & Krug, 1958; Searcy, Craig & Bergquist, 1964; Mischler & Reineke, 1966; Schulman, 1969). Ross (1946) detected proteins migrating as gamma-globulin which he identified as proteases; enzymic degradation of gamma-globulin on storage could explain this result. In contrast others have claimed that human seminal plasma contained a gamma-globulin cross-reacting with that in serum (Klopstock, Hass & Rimon, 1963), and Leithoff & Leithoff (1961) detected IgG, IgA and IgM in human semen using monospecific antisera. Inspection of the immunoelectrophoresis plates of the latter authors reveals that all three lines were in the gammaglobulin position, probably due to contaminant anti-light chain antibodies in the monospecific sera.

Samples of serum and seminal plasma from an infertile man (R)

possessing serum spermagglutinins and an agglutinated ejaculate were generously donated by Dr. D. Israelstam. The results of the analysis of these samples is described.

MATERIALS AND METHODS

Agglutination

Serum R, seminal plasma R, control serum and control seminal plasma were serially diluted in Hank's balanced saline solution containing 0.5% Bovine Serum Albumin and 5% phosphate buffer (pH 7.2). An equal volume of motile human washed spermatozoa was added for a final concentration of $5 \times 10^6$ /ml. The suspensions were incubated at 37°C and scored for agglutination at 30 and 90 minutes.

Mercaptoethanol inactivation of sera

Sera were mixed with an equal volume of 0.2M mercaptoethanol for 24 hours at 4°C, centrifuged and dialysed against phosphate buffer (pH 7.1) or iodoacetate (0.02M). The sera treated with iodoacetate were then further dialysed against phosphate buffer.

Gel filtration on Sephadex G-200

Sera were fractionated as described in chapter 1.

Ion exchange chromatography

1.5 mls of the serum to be fractionated was dialysed against 0.02M phosphate buffer (pH 8.0), and applied to a column of DEAE cellulose 30 x 1cm (Whatman DE52). The serum was fractionated by gradient elution (0.02M to 0.3M) at 15 mls/hr.

Complement fixation

Inactivated sera were serially diluted in duplicate in one volume of barbital complement fixation buffer (Oxoid). One volume of $\frac{1}{20}$ diluted fresh guinea-pig serum (2M.H.D.) was added to each tube. To row (1) of each serum dilution was added one volume of fresh washed human spermatozoa (20 x $10^6$/ml) and to row (2) one volume of fresh human seminal plasma. The tubes were incubated at 37°C for 30 minutes, and 2 volumes of an equal mixture of $\frac{1}{800}$ sheep R.B.C. haemolysin (4 M.H.D.) and a 2% suspension of sheep R.B.C's were added. The tubes were incubated for a further 30 minutes at 37°C and scored for lysis. Controls were (a) no complement (b) no serum (c) no spermatozoa, and (d) normal serum; in controls (a), (b) and (c) one volume of diluent was substituted.

Spermotoxicity

This was performed as described in chapter 2.

Immune fluorescence

Spermatozoa were washed in phosphate buffer (pH 7.1), suspended in test serum for 30 minutes at 37°C, washed three times by centrifugation and resuspension, incubated with fluorescein-conjugated monospecific antisera to human IgA or IgG (Behringwerke) and washed again. Samples were coded, shuffled, mounted in buffered glycerol on slides and scored on the Zeiss

Photomicroscope using dark field fluorescent illumination.

Passive cutaneous anaphylaxis

The procedure was as described in chapter 1.

Immunoelectrophoresis

The micro-method of Scheidegger (1955) was used. Mono-specific antisera to human IgG, IgA and IgM were obtained from Behringwerke. For some tests, the technique of Wadsworth et al (1960) was used. A serum sample was separated by electrophoresis and then allowed to diffuse against an antiserum to it from a trough on one side. A solution of antigens cross reacting with the serum and placed in a trough on the other side of the electrophoresis path also diffused against the antiserum. Any cross-reacting antigens in the solution precipitates along a line between and parallel to the two troughs, and shows complete or partial fusion with the arc between the serum and the antiserum.

Treatment of antigen

Washed human spermatozoa ($20 \times 10^6$/ml) were freeze-thawed 10 times and divided into two parts. One part was centrifuged at 3,200 g for 1 hour and the light supernate and light precipitate removed and stored. The second part was centrifuged at 50,000 g for 1 hour, and the heavy supernate and precipitates removed and stored.

## RESULTS

### Identification of the spermagglutinin in serum and seminal plasma R

Human spermatozoa were agglutinated tail-to-tail by serum R to a titre of $\frac{1}{256}$ and by seminal plasma R to a titre of $\frac{1}{4}$ to $\frac{1}{8}$ (figure 74). The agglutinating activities of both fluids were not destroyed by mercaptoethanol with or without iodoacetate treatment or by heating at 56°C for 60 minutes. Normal sera and seminal plasma failed to agglutinate spermatozoa.

Elution of serum R from Sephadex G-200 produced three fractions which were tested immunoelectrophoretically with antisera to whole serum, IgG, IgM and IgA (figure 75) and for agglutinating activity. Fraction 1, in which only IgM was detected produced no agglutination. Fraction 2 containing IgG and IgA agglutinated to a titre of $\frac{1}{64}$. Fraction 3 containing a trace of IgG agglutinated to a titre of $\frac{1}{16}$.

Gradient elution of serum R from DEAE cellulose produced eleven fractions which were analysed immunoelectrophoretically with antisera to whole serum, IgG, IgM and IgA (figure 76) and for agglutinating activity. The first three fractions, in which only IgG was detected, possessed agglutinating activity. Fractions 4 to 11 did not agglutinate spermatozoa, although IgG was detectable in the first three of these.

Complement was fixed to a titre of $\frac{1}{256}$ in the reaction between serum R and spermatozoa. Normal seminal plasma has anti-complement activity to a titre of about $\frac{1}{4}$ to $\frac{1}{8}$ in the

absence of any serum. The reaction of serum R with normal seminal plasma, and of seminal plasma R with normal spermatozoa did not fix complement at titres greater than $\frac{1}{4}$. The spermotoxicity of serum R did not differ from that of normal human sera.

For PCA tests a guinea-pig antiserum to human spermatozoa and a normal human serum were used as positive and negative controls. The positive control produced strong bluing following intravenous injection of whole semen, washed spermatozoa, fresh seminal plasma and both heavy and light supernates and deposits obtained after freeze-thawing spermatozoa ten times. The negative control serum R and seminal plasma R failed to respond to any of these antigens.

Working with immune fluorescence on human spermatozoa proved difficult but there was clearly no head fluorescence when serum R was followed by conjugated anti IgA or anti IgG, and there was ± tail fluorescence with conjugated anti IgG but not anti IgA. The agglutinating activity of seminal plasma R was removed by absorption with anti IgG but only weakened by absorption with anti IgA.

Evidence that antibodies are transmitted into seminal plasma more readily than into testicular and excurrent ducts

(a) Experiments on man.

The presence and concentrations of immunoglobulins in

twenty samples of normal seminal plasma and in ten samples from infertile men were investigated.

Immunoelectrophoresis of seminal plasma using antiserum to human serum revealed a gamma-globulin line (figure 77) which cross-reacted with serum gamma-globulin (figure 78). Following electrophoretic separation, human serum was allowed to diffuse against an antiserum to human serum forming a gamma-globulin precipitation arc. Autologous seminal plasma in a trough on the other side of the fractionated serum also diffused against the antiserum forming a precipitation line between the two troughs which fused without spurring with the gamma-globulin arc (figure 79).

In contrast to these results, antisera produced in rabbits to human seminal plasma did not contain antibodies to any immunoglobulins (figure 80). Repeated absorption with seminal plasma of antiserum to human serum failed to remove the antibodies to IgG, IgA and IgM present in human serum, but did weaken the precipitating line against some other serum proteins (figure 81).

Monospecific antisera to IgG, IgA and IgM gave single lines against serum, but only the anti $\gamma$-chain serum produced a very weak line against IgG in some samples of seminal plasma (figure 82). If electrophoresis of serum was followed by diffusion against a monospecific antiserum from a trough on one side and against seminal plasma on the other, then a longitudinal precipitation

line was fused with the IgG arc, but not with the IgM arc. There was perhaps a very faint line fusing with some IgA arcs, but it was not possible to exclude light chain contamination in the anti $\alpha$ chain serum.

Pooled human seminal plasma was concentrated 14 fold by volume by lyophilization, and analysed immunoelectrophoretically with monospecific antisera. The presence of IgG but not IgA and IgM was detected.

In human seminal plasma, no IgM could be detected by radial immunodiffusion, which measured as little as 0.03 mgm/ml or about 1.8% of serum levels. IgA was detected in 50% of samples at $0.043 \pm .008$ mgm/ml $\pm$ S.E., which is just above the sensitivity limit of the test and about 1% of the serum. The remaining samples did not contain detectable amounts of IgA. The mean value for seminal plasma IgG was $0.152 \pm .009$ mgm/ml $\pm$ S.E. or about 1% of serum levels. Following concentration of pooled seminal plasma 14 fold by volume, IgM could still not be detected although both IgA and IgG were present.

In all the analyses on human seminal plasma, the samples from normal and infertile men were qualitatively and quantitatively similar.

(b) Experiments on animals.

Immunoelectrophoresis of ovine, bovine and rabbit seminal plasma against antisera to sera from the same species revealed

a gamma-globulin arc in each case. In bull and ram seminal plasma, two gamma-globulins were detected (figures 10 and 83).

The immunoglobulin concentration in ram seminal plasma was measured by radial immunodiffusion as 0.57± 0.04 mgm/ml ± S.E. or about 2% serum values. This is 10 fold greater than the value of 0.04 mgm/ml reported for rete testis fluid in chapter 2.

DISCUSSION

(1) Identification of the spermagglutinin in serum and seminal plasma R.

The distribution of the agglutinating activity in the fractions obtained after ion exchange chromatography and gel filtration of serum R corresponded with that of IgG. This was confirmed by the use of monospecific antisera in the indirect immune fluorescence procedure, which indicated that IgG and not IgA was reacting with spermatozoa. The possession of the activity by IgA or IgM molecules was also excluded by the failure of mercaptoethanol to affect agglutination. IgE molecules are destroyed by heating, but this did not prevent spermagglutination by serum R. Coombs, Edwards & Gurner (personal communication) have since confirmed by the mixed antiglobulin test that the spermagglutination factor in serum R is IgG.

The IgG antibody fixes complement, and this is a property of the IgG1, IgG2 and IgG3 isotypes (Ishizaka et al, 1966) but not of the IgG4 isotype which shows low reactivity with $C'_{1q}$ (Müller-Eberhard, 1968). Complement was fixed when serum R was mixed with whole spermatozoa, with freeze-thawed spermatozoa and with the supernate of freeze-thawed spermatozoa after centrifugation at 3,200 g for 60 minutes but not at 50,000 g for 60 minutes. The antigen on the spermatozoa is not brought into free solution by freeze-thawing.

The IgG antibody failed to give a positive PCA reaction, despite vivid positive controls which indicate that antigen was reaching bound antibody. Human IgG1, IgG3 and IgG4 elicit a PCA response in the guinea-pig but IgG2 does not (Terry, 1965). The antibody responsible for the spermagglutination can therefore be provisionally assigned to the IgG2 isotype and this will be confirmed if the antigen can be brought into free solution. Selective production of IgG2 isotypes is stimulated by immunization with levans and dextrans (Yount et al, 1968). A transition on repeated immunization without adjuvant from antibody type giving positive PCA to one failing to give PCA has been reported for various species (Ovary et al, 1963; Coe 1966). If a similar pattern occurs in the human, a predominantly IgG2 response would eventually result.

The isotype specificities of human IgG are sited on the heavy chain, and the ability of immunoglobulins to cross membranes is also determined by heavy chain structure (Brambell et al, 1960). A differential transmission of human IgG isotypes into prostatic secretions might explain the failure of some serum spermagglutinins to cause infertility. In the case of patient R, the agglutinating antibody does enter seminal plasma and the properties of this antibody resemble those of the serum agglutinin in so far as they could be tested. Coombs, Edwards & Gurner (Personal communication) have confirmed by the mixed antiglobulin test that the main agglutinating activity in

seminal plasma R is possessed by IgG molecules.

The autoantigen which reacts with the IgG antibodies is present on the tail of spermatozoa as shown by agglutination, immune fluorescence and mixed antiglobulin (Coombs et al). It is not destroyed by heating to 100°C and does not readily come into solution. Although complement is fixed in the reaction between the antigen and antibody this does not result in sperm immobilization or lysis.

(2) Relative ease of transmission of antibodies into seminal plasma and testicular secretions.

Immunoglobulins are present in seminal plasma although at levels much lower than in serum. The absence of antibodies to gamma-globulin in antisera induced against seminal plasma and the failure of seminal plasma to absorb out antibodies to immunoglobulins from antiserum to serum, reflect these low levels of immunoglobulin. Nevertheless, the immunoglobulin content of ram seminal plasma was over tenfold higher than that of ram rete testis fluid. The immunoglobulin content of human seminal plasma was close to that of the ram, and examination of the gamma-globulin lines in bovine and rabbit seminal plasma indicates that the immunoglobulin levels in these are similar. The difference between the antibody content of the seminal fluids may be even greater if the seminiferous tubule fluid values are elevated by inflammation at the cannula or leakage of antibody at the more permeable rete testis.

The ratio of the types of immunoglobulin present in human seminal plasma are as expected for simple filtration from serum. Thus IgG and IgA are at 1% of serum levels, and there is even less of the larger IgM. The value for the IgA concentration may be an underestimate if the immunoglobulin is present as a polymer, but even if all the IgA present was polymerised, the levels would still be surprisingly low for an exocrine secretion where IgA usually predominates (Tomasi & Bienenstock, 1968). Exocrine IgA may be wholly or partly synthesized locally, and the failure to find high levels of IgA in seminal plasma could reflect the absence of a mechanism for production of local antibodies. The known autoantigenicity of spermatozoa and seminal plasma proteins would make this desirable. Also, the male genital tract is probably not exposed to as large a quantity of foreign antigen as the respiratory or digestive tracts but, during infection of the genital tract, activation or attraction of cells capable of producing antibody locally might occur. Under such conditions the dangers of autoimmunization with spermatozoal antigen might increase. In many men with circulating autoantibodies to spermatozoa, the IgA serum levels are higher than normal (Rumke, 1969). This could reflect 'overspill' of locally produced antibody.

Finally, it is important to know whether the serum agglutinins that transmit to seminal plasma and cause agglutination of the ejaculate do in fact fail to enter the testis or whether they

enter and are inactive. There is no clinical evidence on this problem, and little from experimental animals. Several authors have reported serum antibodies in male rats and guinea-pigs with normal spermatogenesis, but none have tested for fertility. Repeated low dose injections of spermatozoal suspensions in saline have so far failed to dissociate antibody production from testicular damage in guinea-pigs. The use of pure soluble antigens which is being attempted may prove to be more successful.

## Chapter 7

# THE EFFECT OF IMMUNOLOGICAL BARRIERS IN THE FEMALE GENITAL TRACT ON THE DEPRESSION OF FERTILITY BY SYSTEMIC ANTIBODIES TO SPERMATOZOA.

INTRODUCTION

Systemic immunization of the female with isologous spermatozoa may lead to infertility (see introduction), all or part of which may be due to a direct effect on the spermatozoa prior to fertilization.

It is not known at what point(s) in the female tract the immune response exerts its effect. A large number of spermatozoa are placed in the vagina (or directly into the uterus in many species), but few of these reach the oviduct. In the vagina the immediate environment of the spermatozoa is seminal plasma, and although contact with vaginal secretions will occur with time (Otani et al, 1963; Straus, 1965; Parish et al, 1967) the spermatozoa least likely to be affected by any vaginal antibody will be those that pass up through the uterus to the oviduct.

There is controversy over the antibody content of the uterine secretions, and the extent to which these are transmitted into the uterus from the blood or produced locally. The problem may be approached in two ways, either by collecting and analysing the fluid at oestrus or by measuring the titre of antibody in the fluid to a systemically administered antigen.

The results obtained by the first technique are highly disparate, which undoubtedly reflects the difficulties of fluid collection. The sensitivity of the uterus to trauma casts doubt on the results obtained by squeezing out the fluid with a

hand-wringer (Olds & VanDemark, 1957) collecting fluid from ligated uteri (Stevens, Hafs & Kirton, 1964; Homburger, Grossman & Tregier, 1955) and cannulating the uterus (Menge, 1967). Flushing of uterine horns is usually done on dead uteri, involves the risk of serum contamination when inserting the flushing cannula through the uterine wall, and does not give the actual concentrations of substances merely their total weight per uterus (Heap, 1962). Protein concentrations ranging from about 0.1 gm% to 6.5 gm% have been reported, together with protein compositions including none or most of the serum components. Uterine antibodies to systemically administered antigens have been detected at very low titres or not at all (Edwards, 1960b; Otani et al, 1963; Edwards, 1964) except when there has been evidence of serum contamination (Henle & Henle, 1940: Menge, 1967).

Most of the problems encountered in the analysis of uterine fluid also arise for oviducal fluid for which there are fewer results. They are very similar to those for the uterus both in respect of titres of transmitted antibody which are low (Edwards, 1960b; McLaren, 1964) and protein concentrations which are low but variable (Hamner & Williams, 1965; Holmdahl & Mastroianni, 1965; Marcus & Saravis, 1965; Restall & Wales, 1966).

The composition of fluids from the female tract varies with the cyclic activity of the ovary (Heap & Lamming, 1962), and only samples taken at oestrus will be relevant for studies of anti-

spermatozoal activity. Even more precision in the timing of fluid collection may be necessary, for recently a dramatic change in uterine proteins has been detected at the time of ovulation (Schumacher, 1968). Follicular fluid has a high protein content, and a composition similar to serum (Brambell, 1956; Caravaglios & Cilotti, 1957; Desjardins, Kirton & Hafs, 1966; Lutwak-Mann, 1954; Olds et al, 1957; Shivers, Metz & Lutwak-Mann, 1964; Yatvin & Leathem, 1964; Zachariae & Jensen, 1958). The fluid is presumed on indirect evidence to contain immunoglobulins and antibody activity (Heglar, 1962). Release of this fluid at ovulation could therefore introduce serum proteins into the female tract, and the follicular cell mass will also carry a high protein content fluid which must be penetrated by the few spermatozoa reaching the egg. If antibodies to spermatozoa are present in the female, their maximum antifertility effect should be exerted at this point where the antibody:antigen ration is most favourable.

In this chapter the passage of systemic antibodies into the uterus is reinvestigated, and follicular fluid before and after ovulation is examined.

MATERIALS AND METHODS

Use of uterine diffusion chambers

Uterine diffusion chambers were prepared by Dr. R.G. Edwards for exposing spermatozoa to human or bovine secretions. Each chamber consisted of a nylon tubing frame and a Millipore cellulose ester membrane (figure 84). Experiments in vitro revealed that the membrane (No. RAWP or SSWP) allowed free and rapid passage of all serum proteins. The chambers were loaded with saline, sealed and placed in the oestrous uterus of a conscious woman or cow by means of an I.U.D. inserter tube and rod (figure 85).* The chambers could easily be removed by an attached thread, the end of which lay in the vagina. The fluid in the chamber was withdrawn and analysed.

Estimation of sodium and potassium concentrations

These were done by flame photometry.

Flushing of rabbit uteri

Animals were anaesthetised with nembutal and the uterus and vagina exposed by a mid line ventral incision. The cervices were exposed by an incision in the anterior vaginal wall. One horn was clamped gently at the utero-tubal junction and 0.1 ml of suspension of Indian ink was injected through the uterine wall. The even distribution of the Indian ink through the horn was

* The insertions into women were by Dr. D. Israelstam, Dr.D. Nino or Dr. L. Talbert, and into cows by Dr. L. Rowson.

followed visually. One ml of warm saline was introduced through the cervix by a blunt Pasteur pipette. The saline moved up the horn by uterine contractions and then passed back flushing the fluid contents with it. These were collected by the Pasteur pipette (figures 86 and 87).

Estimation of Indian Ink concentration

A standard solution of Indian ink was prepared in saline. The absorption at 700 mμ of dilutions of this stock solution was measured on the Unicam Spectrophotometer SP500, and a calibration curve was plotted.

Collection of uterine spermatozoa

The oestrous cycles of female guinea-pigs were followed by daily examination for vaginal patency. Oestrous animals were mated twice, and 6-10 hours later the animals were anaesthetised and the uterine horns flushed through the cervices as described for the rabbit. The flushings were immediately diluted in 10 mls of warm buffered saline to dilute any serum contamination. The spermatozoa were concentrated by centrifugation.

Injection of acriflavine

Female oestrous rabbits were anaesthetised with nembutal and given slow intravenous infusions of acriflavine in saline (20 mgm/kgm). Fifteen to thirty minutes later the animals were killed. The uteri, oviducts and ovaries were removed and

samples were snap-frozen in Arcton cooled by liquid nitrogen, freeze-dried and vacuum embedded. Sections were cut, mounted in xylol and examined by dark field fluorescence microscopy.

In some females a solution of acriflavine was injected through the uterine wall into the lumen. Animals were killed fifteen to thirty minutes later, and the uteri snap-frozen and treated as above.

Collection of follicular fluid

Fluid was collected sterilely by puncture of follicles of variable maturity. After removal of the egg and cumulus cells, the fluid was stored at -20°C.

Radial immunodiffusion, immunoelectrophoresis, protein estimation, immune fluorescence and passive cutaneous anaphylaxis were performed as described earlier.

RESULTS

The composition of fluid collected by uterine diffusion chambers

Clear straw-coloured solutions were recovered from the chambers which had been in the human uterus for 12 hours. These solutions were analysed for $Na^+$, $K^+$, $Cl'$ and total protein and were compared with the results of similar analyses of sera collected at the same time. The results for humans are given in tables 16 and 17. Uterine fluid differed in having more $K^+$ and less protein than serum. However, cellulose acetate electrophoresis and immunoelectrophoresis using antiserum to human serum revealed that the protein composition of the fluid and of serum were almost identical (figures 88 and 89). The fluid contained IgG, IgA, IgM and the $\beta_{1C}$ component of complement. Immunization of rabbits with uterine fluid failed to reveal any proteins specific to the fluid.

The results for the cow were essentially similar, although the proportion of protein was slightly less (about 25% that of serum) (Table 18). Analysis of the protein composition of cow uterine fluid gave a result similar to that for humans. No antigens specific to the fluid could be detected by immunizing two rabbits with fluid in adjuvant.

The composition of fluid collected by flushing rabbit uteri

The uterine horns of thirteen oestrous does were flushed as

described in materials and methods. An average volume recovery of 70% of the flushing saline (1.0 ml) was achieved. After flushing, some horns were cut open and Indian ink could be seen trapped on the mucous surface under folds of endometrium. This ink was washed clear and the washings pooled with the flushings; the total ink content matched that of the injected sample showing that negligible losses from the uterine lumen via the cervix or by absorption had occurred.

The expected concentration of Indian ink in the flushings $(C_c)^*$ was calculated from the volume and concentration of the Indian ink injected ($C_1$ and $V_1$) and from the volume of flushing fluid (F). The experimental value ($C_E$) was always lower, and this could have been due either to dilution by uterine fluid or to low flushing efficiency. Simple calculations gave either the uterine fluid volume $(V_u)^*$ assuming 100% flushing efficiency, or the flushing efficiency $(E)^*$ assuming a generous value of about 0.1 ml of fluid per horn.

After measuring the Indian ink content of the flushing at 700 mμ the protein content was measured using absorption at 280 mμ or the Biuret reaction. The value so obtained was corrected for the dilution by flushing fluid. This gave a value for protein concentration assuming 100% flushing efficiency and a large uterine fluid volume. If a further correction for

$$*C_c = \frac{C_1 V_1}{(F+V_1)} \qquad V_u = \frac{C_1 V_1}{C_E} - (V_1 + F) \qquad E = \frac{C_E(F+V_1+0.1)100}{C_1 V_1}$$

flushing efficiency was applied, a value for protein concentration was obtained which assumed a uterine fluid volume of 0.1 ml.

For a flushing efficiency of 100%, the mean protein concentration was 0.81± 0.41 gm% ± S.E. with a mean uterine fluid volume of 0.95± 0.22 mls/horn ± S.E. Assuming a uterine fluid volume of 0.1 ml/horn, the mean protein concentration was 3.32± 1.02 gm% ± S.E. for a flushing efficiency of 71%. The latter values are far more realistic, for the injection of only 0.1 ml of fluid into a horn produces considerable distension, and 1.0 ml of fluid greatly exceeds the volume capacity.

Some flushings were pooled, concentrated ten fold to 'remove' the flushing saline and tested by immunoelectrophoresis against an antiserum to rabbit serum. Only a weak albumin line was detected. If serum proteins were present at a concentration of 1 to 2 gm% more lines should have been apparent.

The transfer of serum antibody activity into uterine fluid

Five female rabbits were immunized with bovine serum albumin in complete Freund's adjuvant to give serum antibody titres of about $\frac{1}{500}$ by PCA in the guinea-pig. The uterine flushings collected from these animals at oestrus were all negative in the same test.

Seven female guinea-pigs were mated and the uterine spermatozoa were collected five hours later by flushing the uterine horns of anaesthetized females. The spermatozoa were tested for a reaction with the natural antibody to spermatozoa

by the direct addition of fluorescein-conjugated anti guinea-pig globulin. No spermatozoa with fluorescent acrosomes were observed, despite the presence of the antibody in the serum to titres of $\frac{1}{64}$.

These results indicate that the ratio of serum protein to uterine fluid protein is much smaller than that of serum antibody activity to uterine fluid antibody activity.

A barrier to acriflavine in the uterus

Acriflavine injected intravenously into oestrous rabbits rapidly stains all uterine cells except the surface epithelial cells adjacent to the uterine lumen (figures 90 and 91). Acriflavine injected into the uterine lumen stains only the epithelial cells (figure 92). A restriction on the diffusion of the dye apparently exists at the base of the epithelial cells. Ovaries taken from animals injected intravenously with acriflavine contained follicles which had stained throughout. Evidently there is no barrier to acriflavine diffusion into the follicles.

The protein composition of follicular fluid

Immunoelectrophoresis of human follicular fluid produced a pattern similar to that of serum but with a weak $\alpha_2$-macroglobulin line and no $\beta_2$- macroglobulin (figure 93). Monospecific antisera revealed the presence of IgA and IgG but not IgM (figure 93). Nine samples of fluid were analysed for the

concentration of various proteins by radial immunodiffusion and the results are presented in table 19. All three immunoglobulins are present, although there is less IgM than IgG or IgA. Immunoelectrophoresis of bovine follicular fluid produced a pattern similar to that for human fluid.

Follicular fluid cannot be collected as such following ovulation. The mass of eggs and cumulus cells which passes into the oviduct can be recovered by simply pricking the oviduct under oil. Cells in an egg mass thus obtained from the golden hamster oviduct were dispersed by exposure to hyaluronidase (0.005 gm%) for 30 minutes and the supernatant removed and analysed immunoelectrophoretically using an antiserum to hamster serum. The result is shown in figure 94. Lines corresponding to albumin and gamma-globulin are present, and the immunoelectrophoretic pattern is similar to that produced by hamster serum diluted $\frac{1}{20}$ to $\frac{1}{40}$.

## DISCUSSION

The high level of serum protein in the human and bovine uterine fluids collected by the diffusion chamber contrasts with the low level of serum protein and antibody found in the rabbit and guinea-pig fluids. The total protein concentrations of both the flushed and chamber uterine fluids were similar, so presumably most of the rabbit proteins were not derived from serum. Beier (1968) has recently reported a similar protein content for rabbit uterine fluid, and has identified most of the protein as specific to the uterus.

The diffusion chamber may have excited a local inflammatory response, producing and then collecting a protein rich exudate (Ascheim & Zweifach, 1962). It has recently been suggested that the I.U.D. acts by provoking a low grade chronic inflammation and leucocytosis in several species (see Corfmann & Segal, 1968). In different regions of the same uterus an inverse correlation was shown between the local inflammation and the ability to give a decidual response (Parr, Schaedler & Hirsch, 1967). The fluid collected in the uterine chambers may not be representative of normal oestrous fluid but of that occurring in the presence of an I.U.D., and provides evidence in favour of this theory of action of the contraceptive device.

The flushings from the rabbit uteri were collected from live, intact animals with minimal trauma, and a rough estimate of the efficiency of the flushing was obtained by the use of a

marker dye. The total protein in the secretion was low and was probably present in a small volume of fluid. The antibody activity of the fluid was even lower. It seems unlikely that the uterine fluids would exert much immunological antifertility effect on the relatively large volume of spermatozoa and seminal plasma placed in the vagina or uterus. Surprisingly there are few studies which test this directly. Edwards (1964) found no agglutination or lysis of spermatozoa in the mouse uterus despite high serum titres. Uterine anaphylaxis was induced by the application of spermatozoa to the peritoneal surface but not the endometrial surface of uteri from guinea-pigs immunized with spermatozoa (Ashitaka, Isojima & Ukita, 1964a,b). Removal of uterine spermatozoa by phagocytosis was slightly increased by immunization, but the rate of phagocytosis was independant of systemic antibody titres (Edwards, 1964; Plank, 1967; Sokolovskaya et al, 1968; Maruta & Moyer, 1965). Symonds (1967) reported that the natural antibody did react with rabbit uterine spermatozoa but only with those which were dead or phagocytosed. The disparity between this result and that described for the guinea-pig may reflect species differences, the time of collection of spermatozoa and also the fact that Symonds flushed dead, dissected uteri.

The available evidence provides little support for any major effect of systemic antibody on uterine spermatozoa. Further, the studies have been performed on uterine spermatozoa

recovered five to ten hours after mating. In most animals which are induced-ovulators or have restricted mating periods, fertilization would have already been achieved by spermatozoa long since present in the oviduct (Yamanaka & Soderwall, 1960; Yanagimachi & Chang, 1963). Uterine spermatozoa may therefore be the wrong spermatozoa to examine. They may also have acted like an I.U.D. inducing an inflammatory exudate containing antibody, and are known to stimulate a leucocytosis (Yanagimachi & Chang, 1962). The uterine environment would not then be representative of that experienced by the fertilizing spermatozoa.

The fertilizing spermatozoon must penetrate the cumulus cell mass, and prolonged contact with these cells and fluids may form part of the process of capacitation (Bavister, 1969; Edwards, Bavister & Steptoe, 1969). The results presented here show that the cumulus mass carries some of its high antibody content down the oviduct. The most potent immunological antifertility effect is likely to be exerted at this point. More direct evidence in favour of this theory can be obtained by comparing the rates of *in vitro* fertilization using egg-cumulus masses from immunized and nonimmunized females.

# CONCLUSION

Many of the problems of immunoreproduction are also those of immunology and of reproductive physiology. The discovery of the heterogeneity of the immune response and its cellular basis, of the dual nature of tolerance and of the delicate and complex relationship between the two which determines the effect of antigen has made many of the problems of immunoreproduction seem less intractable. At the same time, the physiology of reproduction has been reaching the molecular level thus rendering application of immunological techniques more appropriate.

The immunology of spermatozoa, perhaps suffering from its early start and accumulation of unresolved problems, has advanced only falteringly but the problems are now defined and the prospects exciting.

The purification of spermatozoal antigens provides a rather frustrating prerequisite to the potentially exciting developments that could flow from their analysis and use. Already the major stumbling block to an understanding of autoimmune aspermatogenesis posed by the heterogeneity of the immune response is beginning to crumble (Voisin et al, 1969). Controlled induction of one type of immune response by one spermatozoal antigen will doubtless soon resolve the problem completely. Comparison of the qualitative and quantitative responses to pure antigen of mature males with those of virgin females and neonatally castrated males should distinguish whether the immune system recognizes spermatozoa as autologous by natural tolerance, by low-level

paralysis or not at all. If spermatozoa are regarded as 'self', purified antigens modified by controlled procedures could be used to determine which theory of autoimmunity most appropriately describes the response to spermatozoa.

Understanding of the ways in which the immune response interacts with endogenous antigen has also progressed. Experiments in vitro have demonstrated the potent antispermatozoal effects of immune sera. The difficulty in relating this effect to the damage produced in vivo has become less puzzling with the realization that both the spermatozoal antigens and the immune response to them are heterogeneous, and that the contents of the genital tracts are protected by an immunological barrier. The strength of this barrier is remarkable, and comparable with similar barriers isolating, for example, the foetus and the brain. The brain is strikingly similar to the testis for it also contains an autoantigen which develops neonatally after the barrier (Paterson, 1958), secretes a characteristic fluid low in protein and with an immunoglobulin content almost identical to that of rete testis fluid (Chodirker & Tomasi, 1963), shows a reduced barrier efficiency in the inflammatory phase of experimental allergic encephalomyelitis (Barlow, 1956) and shows local lesions at sites where the barrier has been weakened in immunized animals (Clarke & Bogdanove, 1955; Levine & Wenk, 1967). Circulating antibody reportedly depresses immunologically induced brain damage (Paterson, 1966), but although experiments

*in vitro* have suggested a similar effect by PCA antibodies to spermatozoa (Chutna *et al*, 1964), no effect *in vivo* has been described.

Selective transmission of immunoglobulins across membranes and local production of antibody within the genital tracts are topics that assume considerable importance with the discovery of the immunological barrier.

The prospects for immunoreproduction in medical application are no less exciting, but are unlikely to develop rapidly unless more human orientated work is supported. The human infertility resulting from spermagglutinins is far from completely understood, and the causative immune response could be qualitatively or only quantitatively different from that which causes autoimmune aspermatogenesis. Analysis for intratubular antibody in testicular biopsy samples from men with spermagglutinins would provide a rapid answer to this question. It might unfortunately also provide the breach in the testicular barrier which would lead to immunological destruction of the testis. An experimental model for the infertility caused by spermagglutinins is lacking. In the studies described in chapter 3 circulating antibodies to spermatozoa in maturing guinea-pigs were not detected within the seminiferous tubules, but the fertility of these animals could obviously not be tested. Production of a purely humoral response of undetermined type and titre did not cause autoimmune aspermatogenesis in mature guinea-pigs (Brown *et al*, 1967) but

the animals were not tested for fertility or intra-testicular antibody. These studies are well worth pursuing, for this sort of infertility probably provides the best hope at the moment for a contraceptive vaccine.

Azoospermia resulting from immunological damage to the human testis has not yet been reported as such, although experimental isoimmune hypospermatogenesis has been described (Mancini et al, 1965). This form of infertility is unlikely to be of use as a contraceptive technique both because of the likely discomfort and because any process involving massive destruction and absorption of autologous antigen is undesirable, as is illustrated by the alleged generalised autoimmune lesions of vasectomized men (Roberts, 1968). The plight of men hypospermic for immunological reasons demands a search for successful treatment; the possibility of inducing high-level tolerance to spermatozoal autoantigens could be investigated.

There is little ~~uncontradicted~~ unequivocal data on natural or experimental infertility in women immunized with spermatozoa. As a contraceptive technique the approach seems at the moment unlikely to be reliable. The dangers of foetal abnormality suggested by some results (Menge, 1967, 1968, 1969) demand a thorough analysis of antigenic cross-reactivity between spermatozoa and foetal tissue. The discovery and use of heterologous immunogens to induce antibodies reacting with autologous antigens would eliminate cross-reactivity due to blood-group or histocompatibility

antigens.

The immunological barriers themselves have received little direct attention from immunologists. In contrast to isologous antisera, heterologous antisera to rat testis reportedly caused testicular damage on passive transfer (Tyler et al, 1963). Heterologous antisera to kidney were also effective, and both kidney and testis share a basement membrane component which is heteroantigenic (Pierce, 1966). The passively administered antisera may have been weakening the barrier sufficient to allow entry of anti-spermatozoal antibodies. Approaches along these lines using immunological and ~~immuno~~chemical methods less severe than cadmium and more specific than antisera to basement membrane antigens could provide a simple non-surgical method of sterilization.

Although the dissertation has concentrated on spermatozoal antigens, a fascinating array of other antigens are actually present in the reproductive tissues. Some were listed in the Introduction, but many potentially antigenic molecules could be used. Antibodies to blastokinin, uterine lytic factor, capacitation and decapacitation factors or embryonic inducers promise to be useful tools for scientific and medical studies. These antibodies will be unable to exert in vivo effects unless they can reach endogenous antigen, a process which will often require crossing of the immunological barriers of the genital tracts described here.

REFERENCES

Adair, G.S. (1961) The Osmotic Pressure. In A laboratory manual of analytical methods of protein chemistry, 3, 22. Pergamon Press.

Adler, H. (1909) Über Autospermotoxine. Z.Immunitätsforsch., 3, 447.

Ascheim, E and Zweifach, B.W. (1962) Quantitative studies of protein and water shifts during inflammation. Am.J.Physiol., 202, 554.

Asherson, G.L. (1967) The passive transfer of delayed hypersensitivity in the guinea-pig. II. The ability of passively transferred antibody to cause local inflammation and retention of antigen and the role of these phenomena in the passive transfer of delayed hypersensitivity. Immunology, 13, 441.

Asherson, G.L. (1968) The role of micro-organisms in autoimmune responses. Progr. Allerg., 12, 192.

Ashitaka, Y., Isojima, S. & Ukita, H. (1964a) Mechanism of experimental sterility induced in guinea-pigs by injection of homologous testis and sperm. I Relationship between sterility and anaphylactic reaction of the uterus. Fert. Steril., 15, 203-206.

Ashitaka, Y., Isojima, S. & Ukita, H. (1964b) Mechanism of experimental sterility induced in guinea-pigs by injection of homologous testis and sperm. II Relationship between sterility and a spermimmobilizing antibody. Fert.Steril., 15,213

Askonas, B.A. & Rhodes, J.M. (1965a) Immunogenicity of antigen-containing ribonucleic acid preparations from macrophages. Nature, 205, 470.

Askonas, B.A. & Rhodes, J.M. (1965b) Is antigen associated with macrophage RNA? In Molecular and cellular basis of antibody formation, p.503. Ed. J. Sterzl.Prague; Czechoslovak Academy of Sciences.

Barlow, C.F. (1956) A study of abnormal blood-brain permeability in experimental allergic encephalomyelitis. J. Neuropath. Exp.Neurol., 15, 196.

Barros, C. & Austin, C.R. (1967) In vitro fertilization and the sperm acrosome reaction in the hamster. J.Exp.Zool., 166, 317.

Bavister, B.D. (1969) Environmental factors important for

*in vitro* fertilization in the hamster. J.Reprod.Fert., 18, 544.

Beck, J.S., Edwards, R.G. & Young, M.R.(1962) Immune fluorescence technique and the isoantigenicity of mammalian spermatozoa. J. Reprod. Fert., 4, 103.

Bedford, J.M. (1965) Effect of environment on phagocytosis of rabbit spermatozoa. J.Reprod.Fert., 9, 249.

Bedford, J.M.(1969) In Mechanisms involved in conception. Schering Symposium, Berlin. In press.

Beernink, K.D. & Steward, J.P. (1968) The adherence to Salmonella enteritidis of IgG, IgM and $\beta_{1C}$-globulins from guinea-pig serum. Immunol., 15, 319.

Behrman, S.J. & Nakayama, M. (1965) Antitestis antibody: its inhibition of pregnancy. Fert.Steril., 16, 37.

Behrman, S.J. & Otani, Y.(1963) Transvaginal immunization of the guinea-pig with homologous testis and epididymal sperm. Int.J.Fert., 8, 829.

Beier, H.M.(1968) Biochemisch-entwicklungsphysiologische Untersuchungen am Proteinmileau für die Blastozystentwicklung des Kaninchens (Oryctolagus cuniculus). Zool.Jb.Anat.Bd., 85, 72.

Beiser, S.M., Erlanger, B.F., Agate, F.J. & Liberman, S.(1959) Antigenicity of steroid-protein conjugates. Science, N.Y., 129, 564.

Bell, E.B. (1968) An immune-type agglutination of mouse spermatozoa by Pseudomonas Maltophilia. J.Reprod.Fert.,17, 275.

Bennett, S.H., Luft, J.M. & Hampton, J.C.(1959) Morphological classification of vertebrate blood capillaries. Amer.J.Physiol., 196, 381.

Berenblum, I., Burger, M. & Knyszynski, A.(1968) Regeneration of bone marrow cells and thymus induced by 19S $\alpha_2$-globulin in irradiated mice. Nature, Lond., 217, 857.

Bishop, D.W. (1961) Aspermatogenesis induced by testicular antigen uncombined with adjuvant. Proc.Soc.Exp.Biol.Med., 107, 116.

Bishop, D.W. & Carlson, G.L.(1965) Immunologically induced aspermatogenesis in guinea-pigs. Ann.N.Y.Acad.Sc., 124, 247.

Bishop, D.W., Narbaitz, R. & Lessof, M. (1961) Induced aspermatogenesis in adult guinea-pigs injected with testicular antigen and

adjuvant in neonatal stages. Devel.Biol., 3, 444.

Bloch, K.J., Kourilsky, F.M., Ovary,Z.& Benacerraf, B.(1963). Properties of guinea-pig 7S antibodies. III. Identification of antibodies involved in complement fixation and haemolysis. J.Exp.Med., 117, 965.

Boettcher, B.(1967) Are the genes in spermatozoa functional? Int.Symp.Immun.Spermatozoa and Fertilization, Varna,p.74. Bulgarian Academy of Sciences.

Boettcher, B.(1969) Blood group antigens in seminal plasma, and the nature of human sperm-coating antigen. In Immunology and Reproduction, p.148. Ed.R.G. Edwards. International Planned Parenthood Federation.

Boquet, A. & Bretey, J. (1934) Développement et évolution de la sensibilité à la tuberculine chez le cobaye. Ann.Inst. Pasteur, 52, 252.

Boughton, B. & Spector, W.G.(1963) Autoimmune testicular lesions induced by injury to the contralateral testis and intradermal injection of adjuvant. J.Path.Bact., 86, 69.

Boyden, S.V.(1962a) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J.Exp.Med., 115, 453.

Boyden, S.V. (1962b) Cellular discrimination between indigenous and foreign matter. J.Theoret.Biol., 3, 123.

Boyden, S.V.(1966) Natural antibodies and the immune response. Adv.in Immunol., 5, 1.

Brambell, F.W.R. (1956) Chap.5 in Marshall's Physiology of Reproduction. ed. A.S. Parkes, Longmans, Green, London.

Brambell, F.W.R., Hemmings, W.A., Oakley, C.L. & Porter, R.R. (1960) The relative transmission of the fractions of papain hydrolysed homologous γ-globulin from the uterine cavity to the foetal circulation in the rabbit. Proc.Roy.Soc.B., 151, 478.

Bratanov, K. (1969) Antibodies in the reproductive processes in the female. Immunology and Reproduction, p. 175. Ed. R.G. Edwards. International Planned Parenthood Federation.

Bratanov, K., Dikov, V. & Popova, Y. (1964) On the formation of autospermantibodies in blood animals. Dokl.Bulg.Acad.Nauk., 12, 1117.

Brown, P.C., Glynn, L.E. & Holborow, E.J.(1963) The pathogenesis of experimental allergic orchitis in guinea-pigs. J.Path.Bact., 86, 505.

Brown, P.C., Glynn, L.E. & Holborow, E.J. (1967). The dual necessity for delayed hypersensitivity and circulating antibody in the pathogenesis of experimental allergic orchitis, in guinea-pigs. Immunology, 13, 307.

Brown, P.C., Holborow, E.J. & Glynn, L.E. (1965). The aspermatogenic antigen in experimental allergic orchitis in guinea-pigs. Immunology, 9, 255.

Burnet, F.M. (1959) The clonal selection theory of acquired immunity. Cambridge University Press.

Burnet, F.M. & Holmes, M.C. (1965) Genetic investigations of autoimmune disease in mice. Nature, Lond., 207, 368.

Busey, W.M. (1965) Immunologically induced testicular degeneration in rams. Ph.D. thesis. Colorado State University.

Caravaglios, R. & Cilotti, R. (1957) A study of the proteins of the follicular fluid of the cow. J. Endocr., 15, 273.

Chang, M.C. (1947) The effects of serum on spermatozoa. J. gen. Physiol., 30, 321.

Chiquoine, A.D. (1964) Observations on the early events of cadmium necrosis of the testis. Anat. Rec., 149, 23.

Chodirker, W.D. & Tomasi, T.B. (1963) Gamma-globulins: quantitative relationships in human serum and non-vascular fluids. Science, 142, 1080.

Chutna, J. & Rychlikova, M. (1964) A study of the biological effectiveness of antibodies in the development and prevention of experimental autoimmune aspermatogenesis. Folia Biol., 10, 188

Clarke, G. & Bogdanove, L.M. (1955) The induction of the lesions of allergic meningoencephalomyelitis in a predetermined location. J.Neuropath.Exp.Neurol., 14, 433.

Clegg, E.J. & Carr, I. (1966) Increased vascular permeability in the reproductive organs of cadmium chloride treated male rats. J.Anat., 100, 696.

Clegg, E.J. & Carr, I. (1967) Changes in the blood vessels of the rat testis and epididymis produced by cadmium chloride. J.Path. Bact., 94, 317.

Clegg, E.J., Niemi, M. & Carr, I. (1969) The age at which the blood vessels of the rat testis become sensitive to cadmium salts. J.Endocr., 43, 445.

Coe, J.E.(1966) 7S $\gamma_1$ and 7S $\gamma_2$ antibody response in the mouse.

I.Influence of strain, antigen and adjuvant. J. Immunol., 96, 744.

Cohen, S. & Milstein, C. (1967) Structure and biological properties of immunoglobulins. Adv.in Immunol., 7, 1.

Cohen, S.C., Sapp, T.M., Rizzo, A.P. & Kostage, S.T. (1964). Experimental eosinophilia VII. Lymph node responses to altered globulins. J.Allerg., 35, 346.

Corfmann, P.A. & Segal, S.J. (1968) Biologic effects of intra-uterine devices. Am.J.Obstet.Gynec., 100, 448.

Cowie, A.T., Lascelles, A.K. & Wallace, J.C. (1964) The flow and protein content of testicular lymph in conscious rams. J. Physiol., Lond., 171, 176.

Crabo, B. (1963) Fine structure of the interstitial cells of the rabbit testis. Z.Zellforsch. mikrosk.Anat., 61, 587.

Dempsey, E.W., Greep, R.O. & Deane, H.W. (1949) Changes in the distribution and concentration of alkaline phosphatases in tissues of the rat after hypophysectomy or gonadectomy, and after replacement therapy. Endocr., 44, 88.

Desjardins, C., Kirton, K.T. & Hafs, M.D. (1966) Some chemical, immunochemical and electrophoretic properties of bovine follicular fluid. J.Reprod. Fert., 11, 237.

Dixon, F.J. (1968) Aetiology of glomerulonephritis. Amer.J.Med., 44, 493.

Drevius, L.O. (1968) On the mechanism of sperm immobilization induced by normal sera and anti-semen sera. Exp.Cell Research, 51, 362.

Duke-Elder, S. (1966) System of Ophthalmology. Vol. IX, Diseases of the uveal tract. Kimpten, London.

Dukes, C.D. & Franklin, R.R. (1968) Sperm agglutinins and human infertility: female. Fert.Steril., 19, 263.

Edwards, R.G. (1960a) Complement-fixing activity of normal rabbit serum with rabbit spermatozoa and seminal plasma. J.Reprod.Fertil., 1, 268.

Edwards, R.G. (1960b) Antigenicity of rabbit semen, bull semen and egg yolk after intravaginal or intramuscular injections into female rabbits. J.Reprod.Fert., 1, 385.

Edwards, R.G. (1964) Immunological control of fertility in female mice. Nature, Lond., 203, 50.

Edwards, R.G. (1967) Antibodies and Fertility. Science Journal (London), 3, 69.

Edwards, R.G. (1969) Transmission of antibodies across membranes of the reproductive tracts. Immunology and Reproduction, p.28. Ed. R.G. Edwards. International Planned Parenthood Federation.

Edwards, R.G., Bavister, B.D. & Steptoe, P.C. (1969) Early stages of fertilization in vitro of human oocytes matured in vitro. Nature (Lond.), 221, 632.

Edwards, R.G., Ferguson, L.C. & Coombs, R.R.A. (1964) Blood group antigens on human spermatozoa. J.Reprod.Fert., 7, 153.

Eyquem, A. (1969) Classification and properties of antibody molecules. In Immunology and Reproduction, p.3, Ed. R.G. Edwards. International Planned Parenthood Federation.

Ferin, M., Zimmering, P.E., Lieberman, S. & Vande Wiele, R.L., (1968) Inactivation of the biological effects of exogenous and endogenous estrogens by antibodies to 17β - Estradiol. Endocr., 83, 565.

Fjallbrandt, B. (1967) Fertility in man autoimmunized to sperm. J.Reprod.Fert., 14, 143.

Fjallbrandt, B. (1968a) Sperm agglutinins in sterile and fertile men. Acta Obst.Gynec.Scand., 47, 1.

Fjallbrandt, B. (1968b) Sperm antibodies and sterility in men. Acta Obstet.Gynec.Scand., 47, Suppl.4, p.38.

Flickinger, C. & Fawcett, D.W. (1967) The junctional specialization of Sertoli cells in the seminiferous epithelium. Anat.Rec., 158, 207.

Freund, J., Lipton, M.M. & Thompson, G.E. (1953) Aspermatogenesis in the guinea-pig induced by testicular tissue and adjuvant. J.Exp.Med., 97, 711.

Freund, J., Lipton, M.M. & Thompson, G.E. (1954) Impairment of spermatogenesis in the rat after cutaneous injection of testicular suspension with complete adjuvant. Proc.Soc.Exp. Biol.Med., 87, 408.

Freund, J., Thompson, G.E. & Lipton, M.M. (1955) Aspermatogenesis,

anaphylaxis and cutaneous sensitization induced in the guinea-pig by homologous testicular extract. J.Exp.Med., 101, 591.

Goel, V.D., Soni, B.K. & Bhalla, R.C. (1967) Studies of the effect of hyperimmunization with semen-antigens on reproduction and fertility in female goats. J.Reprod.Fert., 14, 530.

Grey, S. & Huggins, C. (1942) Electrophoretic analysis of human semen. Proc.Soc.Exp.Biol., 50, 351.

Gullbring, B. (1957) Investigation on the occurrence of blood group antigens in spermatozoa from man and serological demonstration of the segregation of characters. Acta Med. Scand., 159, 169.

Gunn, S.A., Gould, T.C. & Anderson, W.A.D. (1963) The selective injurious response of testicular and epididymal blood vessels to cadmium and its prevention by zinc. Am.J.Path., 42, 685.

Halpern, B.N., Ky, T. & Robert, B. (1967) Clinical and immunological study of an exceptional case of reaginic type sensitization to human seminal fluid. Immunol., 12, 247.

Hamner, C.E. & Williams, W.I. (1965) Composition of rabbit oviduct secretions. Fert.Steril., 16, 170.

Heap, R.B. (1962) Some chemical constituents of uterine washings - a method of analysis with results from various species. J.Endocr., 24, 367.

Heap, R.B. & Lamming, G.E. (1962) The influence of ovarian hormones on some chemical constituents of the uterine washings of the rat and rabbit. J. Endocr., 25, 57.

Heglar, R. (1962) Relationship between follicular fluid ABO(H) systems and fertility. Int.J.Fert., 7, 321.

Heidger, P.M. (1969) An electron microscopic study of a barrier to the entrance of horseradish peroxidase into the seminiferous tubule. Anat.Rec., 163, 198.

Heim, W.G. (1968) Relation between rat slow $\alpha_2$-globulin and $\alpha_2$-macroglobulin of other mammals. Nature, Lond., 217, 857.

Henle, W. & Henle, G., (1940) Spermatozoal antibodies and fertility. II Attempt to induce temporary sterility in female guinea-pigs by active immunization against spermatozoa. J. Immunol., 38, 105.

Hermann, G., Licht, W., Keutel, H.J. & Krug, E. (1958) Zu Frage der Identitat von menschlichen spermplasmaprotein und Serumeiweissen. Naturwissenschaften, 11, 268.

Hirsch, J.G. (1965) Neutrophil and eosinophil leucocytes. In The inflammatory process. Ed. by B.W.Zweifach, L. Grant and R.T. McClusky. Academic Press.

Holborow, E.J., Brown, P.C., Glynn, L.E., Hawes, M.D., Gresham, G.A., O'Brien, T.F. & Coombs, R.R.A. (1960) The distribution of the blood group A antigen in human tissues. Brit.J. Exp.Path., 41, 430.

Holmdahl, T.H. & Mastroianni, L. (1965) Continuous collection of rabbit oviduct secretions at low temperature. Fert.Steril., 16, 587.

Homburger, F., Grossman, M.S. & Tregier, A. (1955) Experimental hydro-uteri (Hydrometra) in rodents and some factors determining their formation. Proc.Soc.Exp.Biol.Med., 90, 719.

Hunter, A.G. & Hafs, H.D.(1964) Antigenicity and cross-reactions of bovine spermatozoa. J.Reprod.Fertil., 7, 357.

Hunter, A.G. & Hafs, H.D. (1965) Differentiation of sperm protein from that of seminal plasma in rabbits. Fed.Proc.(Abstr.), 24, 2700.

Imbabi, S.E. (1967) Experimental autoallergic aspermatogenesis in the rat. Dissertation for Ph.D. Michigan State University.

Irvine, W.J. (1964) Thyroid auto-immunity as a disorder of immunological tolerance. Quart.J.Exp.Physiol., 49, 324.

Irvine, W.J., Chan, M., Scarth, L., Kolb, F.O., Hartog, M., Bayliss, R.I.S. & Drury, M.I. (1968) Immunological aspects of premature ovarian failure associated with idiopathic Addison's disease. Lancet, ii, 883.

Ishizaka, T., Ishizaka, K., Salmon, S. & Fudenberg, H. (1966) Biologic activities of aggregated immunoglobulins of different classes. Fed.Proc., 25, 489.

Isojima, S., Graham, R.M. & Graham, J.B. (1959) Sterility in female guinea-pigs induced by injection with testis. Science, N.Y., 129, 44.

Isojima, S. & Li, T.S. (1968) Stepwise appearance of sperm-specific antigens in rats and their disappearance after fertilization. Fert.Steril., 19, 991.

Israelstam, D.M. (1969) The incidence of spermagglutinating

antibodies in the sperm of infertile women. Fert.Steril., 20, 275.

Jerne, N.K. (1955) The natural-selection theory of antibody formation. Proc.Natl.Acad.Sci. U.S., 41, 849.

Johnson, M.H. (1969) The effect of $CdCl_2$ on the blood-testis barrier of the guinea-pig. J.Reprod.Fertil., 19, 551.

Johnson, M.H. & Setchell, B.P. (1968) Protein and Immunoglobulin content of rete testis fluid of rams. J.Reprod.Fertil., 17, 403.

Jonas, W.E., Gurner, B.W., Nelson, D.S. & Coombs, R.R.A. (1965) Passive sensitization of tissue cells. Int.Arch.Allergy, 28, 86.

Kaplan, M.H. (1960) The concept of autoantibodies in rheumatic fever and in the post-commissurotomy state. Anu.N.Y.Acad.Sci., 86, 974.

Kaplan, M.H. (1965) Induction of autoimmunity to heart in rheumatic fever by streptococcal antigens cross-reactive with heart. Fed.Proc., 24, 109.

Karsznia, R., Spielman, W. & Trinkaus, J. (1969) Zum Nachweis der Blutgruppenantigene A und B am menschlichen Spermien. Gynaecologia, 167, 14.

Katsh, S. (1959) Infertility in female guinea-pigs induced by injection of homologous spermatozoa. Am.J.Obstet.Gynec., 78, 276.

Katsh, S. (1960) Localization and identification of autispermatogenic factor in guinea-pig testicles. Int.Arch.Allergy., 16, 241.

Katsh, S. (1964) Adjuvants and aspermatogenesis in the guinea-pig. Int.Arch.Allergy, 24, 319.

Katsh, S. & Bishop, D.W. (1958) The effects of homologous testicular and brain and heterologous testicular homogenates combined with adjuvant upon the testes of guinea pigs. J.Embryol.Exp.Morph., 6, 94.

Kidd, J.G. & Friedewald, W.F. (1942) A natural antibody that reacts in vitro with a sedimentable constituent of normal tissue cells. J.Exp.Med., 76, 557.

Kirkpatrick, C.H. & Katsh, S. (1964) Amino-acid content of

antispermatogenic antigen. Nature, 201, 197.

Klopstock, A., Haas, R. & Rimon, A. (1963) Immunoelectrophoretic analysis of seminal plasma. Fert.Steril., 14, 530.

Kormano, M. (1967a) Dye permeability and alkaline phosphatase activity of testicular capillaries in the postnatal rat. Histochemie, 9, 327.

Kormano, M. (1967b) Distribution of injected L-3, 4-Dihydroxyphenylalanine (L-Dopa) in the adult rat testis and epididymis. Acta Phys.Scand., 71, 125.

Kormano, M. (1968) Penetration of intravenous trypan blue into the rat testis and epididymis. Acta histochem., 30, 133.

Kormano, M. & Penttilä, A.(1968). Distribution of endogenous and administrated 5-Hydroxytryptamine (5HT) and dopamine (DA) in the rat testis and epididymis. Scand.J.Clin.Lab. Invest., 21, Suppl. 101, 93.

Krieg, H. (1967) Blood group substances and infertility. Int.Symp.Immun.Spermatozoa and Fertilization, Varna, 1967. Bulgarian Academy of Sciences.

Lachmann, P.J., Sell, K.W. & Spooner, R.L. (1965) The mixed conglutination reaction. Immunol., 8, 345.

Lacy, B. (1967) The seminiferous tubule in mammals. Endeavour, 27, 101.

Laurence, K.A. (1962) In proceedings of a conference on immunoreproduction. Population Council., p.29.

Leeson, T.S. (1962) Electron microscopy of the rete testis of the rat. Anat.Rec., 144, 57.

Leithoff, H. & Leithoff, I. (1961) Der Nachweis von Bluteiweisskorpen in menschlichen samenplasma mit Hilfe der Immunoelektrophorese. Forschung und Praxis, 21, 1137.

Lerner, R.A. & Dixon, F.J. (1966) Transfer of ovine experimental allergic glomerulonephritis (EAG) with serum. J.Exp.Med., 124, 431.

Levine, P. & Celano, M.J. (1961) The question of D (Rh) antigenic sites on human spermatozoa. Vox.Sang., 6, 720.

Levine, S. & Wenk, E.J. (1967) Rapid passive transfer of allergic encephalomyelitis. J.Immunol., 99, 1277.

Lewis, J.H. (1933) The immunological specificity of brain tissue. J.Immunol., 24, 193.

Lewis, J.M. (1934) The antigenic relationship of the alcohol soluble fractions of brain and testicle. J.Immunol., 27, 473.

Leiberman, S., Erlanger, B.F., Beiser, S.M. & Agate, F.J., Jr. (1959) Aspects of steroid chemistry and metabolism. Steroid-protein conjugates: their chemical, immunochemical and endocrinological properties. Recent Progress in Hormone Research, 15, 165.

Linzell, J.L. & Setchell, B.P. (1968) The output of spermatozoa and fluid by, and the metabolism of, the isolated perfused testis of the ram. J.Physiol., 195, 25P.

Litt, M. (1963) Studies on experimental eosinophilia V. Eosinophils in lymph nodes of guinea-pigs following primary antigenic stimulation. Am.J.Path., 42, 529.

Lumsden, T. (1929) Natural cytotoxins (heterotoxins). Protection of cells against homologous antibodies. J.Path.Bact., 32, 185.

Lutwak-Mann, C. (1954) Note on the chemical composition of bovine follicular fluid. J.Agric.Sci.,Camb., 44, 477.

Majsky, A. & Hraba, T. (1960) Demonstration of D (Rh) agglutinogens in human spermatozoa. Folio Biol., Praha, 6, 342.

Mäkelä, O., Ruoslahti, E. & Enholm, C. (1969) Subtypes of human ABO blood groups and subtype-specific antibodies. J.Immunol., 102, 763.

Mancini, R.E., Andrada, J.A., Saraceni, D., Bachmann, A.E., Lavieri, J.C. & Nemirovsky, M. (1965) Immunological and testicular response in man sensitized with human testicular homogenate. J.Clin.Endocr., 25, 859.

Mancini, R.E., Vilar, O., Alvarez, B. & Sieguer, A.C. (1965) Extravascular and intratubular diffusion of labelled serum proteins in the rat testis. J.Histochem.Cytochem., 13, 376.

Marcus, S.L. & Saravis, C.A. (1965) Oviduct fluid in the rhesus monkey: a study of its protein components and its origin. Fert.Steril., 16, 785.

Maruta, H. & Moyer, D.L. (1965) Phagocytosis of spermatozoa in immunized and non-immunized female guinea-pigs. Fed.Proc., 24, 450.

Maruta, H. & Moyer, D.L. (1967) Immunologic studies of the antigens of guinea-pig semen. Fert.Steril., 18, 649.

Mason, K.E. & Young, J.O. (1967) Effects of cadmium upon the excurrent duct system of the rat testis. Anat.Rec., 159, 311.

Matousek, J. (1964) Antigenic characteristics of spermatozoa from bulls, rams and boars II. sperm agglutinins in normal and immune serum. J. Reprod.Fert., 8, 5.

McLaren, A. (1964) Immunological control of fertility in female mice. Nature, Lond., 201, 582.

McClaren, A. (1966) Studies in the isoimmunization of mice with spermatozoa. Fert.Steril., 17, 492.

Menge, A.C. (1967) Induced infertility in cattle by isoimmunization with semen and testis. J.Reprod.Fert., 13, 445.

Menge, A.C. (1968) Fertilization, embryo and fetal survival rates in rabbits isoimmunized with semen, testis and conceptus. Proc.Soc.Exp.Biol.Med., 127, 1271.

Menge, A.C. (1969) Survival of rabbit blastocysts after incubation with isoantisera against spermatozoa and seminal plasma. In Immunology and Reproduction, Ed.R.G. Edwards. International Planned Parenthood Federation.

Menge, A.C. & Protzman, W.P. (1967) Origin of the antigens in rabbit semen which induce antifertility antibodies. J.Reprod.Fert., 13, 31.

Metalnikoff, S. (1900) Etudes sur la spermatoxine. Ann.Inst. Pasteur, 14, 577.

Metchnikoff, E. (1900) Récherches sur l'influence de l'organisme sur les toxines. Annls.Inst.Pasteur,Paris, 14, 1.

Mischler, T.W. & Reineke, E.P. (1966)Some electrophoretic and immunological properties of human semen. J.Reprod.Fert., 12, 125.

Moller, E. & Moller, G. (1962) Quantitative studies of the sensitivity of normal and neoplastic cells to the cytotoxic action of isoantibodies. J.Exp.Med., 115, 527.

Monastirsky, R. & Fernandez Collazo, E.L. (1967) Cytotoxic effects of normal sera on germinal cells of the guinea-pig. Acta Phys.Latinoamer., XVII, 130.

Monesi, V. (1965) Synthetic activities during spermatogenesis in the mouse. Exp.Cell Research, 39, 197.

Moyer, D.L. & Maruta, H. (1967) Induced isoantibody to homologous seminal and spermatozoal antigens in female monkeys. Fert.Steril., 18, 497.

Müller-Eberhard, H.J. (1968) Chemistry and reaction mechanisms of complement. Advances in Immunol., 8, 1.

Müller-Eberhard, H.J. & Lepow, I.H. (1965) C'1 esterase effect on activity and physicochemical properties of the fourth component of complement. J.Exp.Med., 121, 819.

Murakami, M. (1966) Elektronmikroskopische Untersuchungen um interstitellen Gewebe des Rattenhodens, unter Berücksichtigung der Leydigschen Zwischenzellen. Z.Zellforsch., 72, 139.

Nairn, R.C. (1964) In Fluorescent protein tracing. G. and S. Livingstone Ltd.

Nakamura, R.M. & Weigle, W.O. (1969) Transfer of experimental autoimmune thyroiditis by serum from thyroidectomised donors. J.Exp.Med., 130, 263.

Nelson, D.S. (1968) Macrophages and Immunity. North-Holland.

Nelson, R.A. & Lebrun, J. (1956) The requirement for antibody and complement for in vitro phagocytosis of starch granules. J.Hyg., Camb., 54, 8.

Niemi, M. & Kormano, M. (1965) An angiographic study of cadmium-induced vascular lesions in the testis and epididymis of the rat. Acta Path. Microbiol.Scand., 63, 513.

Olds, D. & Van DeMark, N.L. (1957) Composition of luminal fluids in bovine female genitalia. Fert. Steril., 8, 343.

Ortavant, R., Courot, M. & Hochereau, M.T. (1969) In Reproduction in Domestic Animals. Ed.H.H. Cole and P.T. Cupps. Academic Press.

Otani, Y. & Behrman, S.J. (1963) Immunization of the guinea-pig with homologous testis and sperm. Fert.Steril., 14, 456.

Ovary, Z., Benacerraf, B. & Bloch, K.J. (1963) Properties of guinea-pig 7S antibodies. II Identification of antibodies involved in passive cutaneous and systemic anaphylaxis. J.Exp.Med., 117, 951.

Parish, W.E., Carron-Brown, J.A. & Richards, C.B. (1967). The detection of antibodies to spermatozoa and to blood group antigens in cervical mucus. J.Reprod.Fert., 7, 163.

Parizek, J. (1960) Sterilization of the male by cadmium salts. J.Reprod.Fert., 1, 294.

Parkes, A.S. (1966) The internal secretions of the testis. In. Marshall's Physiology of Reproduction Third Edition. Ed. A.S. Parkes, Longmans.

Parr, E.L., Schaedler, W.R. & Hirsch, J.G. (1967) The relationship of polymorphonuclear leukocytes to infertility in uteri containing foreign bodies. J.Exp.Med., 126, 523.

Parsons, E.I. & Hyde, R.R. (1940) An evaluation of spermatotoxic sera in the prevention of pregnancy. Am.J.Hyg.B., 31, 81.

Paterson, P.Y. (1958) Studies of immunological tolerance to nervous tissue in rats. Ann.N.Y.Acad.Sci., 73, 811.

Paterson, P.Y. (1966) Experimental allergic encephalomyelitis and autoimmune disease. Adv.in Immunol., 5, 131.

Peleg, B.A. & Ianconescu, M. (1966) Spermagglutination and spermadsorption due to Myxoviruses. Nature, Lond., 211, 1211.

Pernot, E. (1956) Récherches sur les constituants antigèniques des spermatozoides des cobayes. Bull.Soc.Chim.Biol. (Paris), 38, 1041.

Phadke, A.M. & Padukone, K. (1964) Presence and significance of autoantibodies against spermatozoa in the blood of men with obstructed vas deferens. J.Reprod.Fert., 7, 163.

Pierce, G.B. (1966) The development of basement membranes of the mouse embryo. Devel.Biol., 13, 231.

Plank, S.J. (1967) Mechanisms of experimental immunofertility. In Proc. 8th Int.Conf.IPPF, Santiago, p. 430, London, 1967. International Planned Parenthood Federation.

Pokorna, Z., Vojtiskova, M., Rychlikova, M. & Chutna, J. (1963). An isologous model of experimental autoimmune aspermatogenesis in mice. Folio Biol., Praha, 9, 203.

Popivanov, R., Sturkalev, I., Evrev, T. & Ananiev, T. (1966). Group antigens in the spermatozoa of non-secretors. Akusherstvo i Ginekol, Sofia, 4, 237.

Popivanov, R. & Vulchanov, V.H. (1961) Identity and differences in the behaviour of species antigens in man's spermatozoa and erythrocytes. C.r.Acad.Bulg.Sci., 14, 223.

Popivanov, R. & Vulchanov, V.H. (1962a) Segregation of man's AB-group spermatozoa in A- and B- spermatozoa through agglutination with immune rabbit anti-A serum. Z.Immun. Forsch.exp.Ther., 124, 206.

Popivanov, R. & Vulchanov, V.H. (1962b) On the character and inter-relations of the species-specific antigens (agglutinogens) of human spermatozoa and erythrocytes. Izvestija Inst. Microbiol.Bulg.Acad.Sci., 13, 81.

Popivanov, R. & Vulchanov, V.H. (1965) Organ antigens in human spermatozoa. Bull.Exp.Biol.Med., Moscow, 2, 110.

Quinlivan, W.L.G. & Masouredis, S.P. (1962) Rho(d) antigen content of human spermatozoa. Immunology, 5, 267.

Quinn, P.J., White, I.G. & Cleland, K.W. (1969) Chemical and ultrastructural changes in ram spermatozoa after washing, cold shock and freezing. J.Reprod.Fert., 18, 209.

Rabinovitch, M. (1967a) The role of antibodies in the ingestion of aldehyde-treated erythrocytes attached to macrophages. J.Immunol., 99, 232.

Rabinovitch, M. (1967b) Studies on the immunoglobulins which stimulate the ingestion of gluteraldehyde-treated red cells attached to macrophages. J.Immunol., 99, 1115.

Raitsina, S.S. & Nilovsky, M.N. (1967) Post-traumatic aspermatogenesis. Resemblance of pathogenesis of autoallergic and post-traumatic orchitis. Folia Biol., Praha, 9, 203.

Raj K. Gupta, Barnes, G.W. & Skelton, F.R. (1967) Light microscopic and immunopathologic observations on $CdCl_2$ induced injury in mature rat testis. Am.J.Path., 51, 191.

Rao, S.S. (1969) Antibodies against hormones. Immunology and Reproduction, P. 63, Ed. R.G. Edwards. International Planned Parenthood Federation.

Rao, S.S. & Sadri, K.K. (1959) Immunological studies with human semen and cervical mucus. Proc.Sixth.Int.Conf.I.P.P.F., New Delhi, p.313, Excerpta Medica.

Rao, S.S. & Sadri, K.K. (1960) The antigenic composition of buffalo semen. J.Comp.Path.Ther., 70, 1.

Rao, S.S., Sadri, K.K. & Sheth, A.R.(1961) The antigenicity of human spermatozoa and its significance. Proceedings of the Symposium on Proteins. C.F.T.R.I. Mysore.

Reisfeld, R.A. & Hyslop, N.E. (1966) Separation of 7S $\gamma_1$-globulin, 7S $\gamma_2$-globulin and macroglobulin guinea-pig antibodies. Proc.Soc.Exp.Biol.Med., 121, 508.

Restall, B.J. & Wales, R.G. (1966) The fallopian tube of the sheep III The chemical composition of the fluid from the fallopian tube. Aust.J.Biol.Sci., 19, 687.

Roberts, H.J. (1968) Voluntary sterilization in the male. Brit.Med.J., 17th August, 434.

Roosen-Runge, E.C.(1962) The process of spermatogenesis in mammals. Biol.Rev., Cambridge Phil.Soc., 37, 342.

Rosenthal, L. (1931) Spermagglutination by bacteria. Proc.Soc. Exp.Biol.Med., 28, 827.

Ross, M.H. (1963) The fine structure of testicular interstitial cells in the rat. Anat.Rec., 145, 277.

Ross, M.H. (1967) The fine structure and development of the peritubular contractile cell component in the seminiferous tubules of the mouse. Am.J.Anat., 121, 523.

Ross, V. (1946) Precipitin-reactions of human seminal plasma. J.Immunol., 52, 87.

Roussel, J.D., Stallcup, O.T. & Austin, C.R.(1967) Selective phagocytosis of spermatozoa in the epidiymis of bulls, rabbits and monkeys. Fert.Steril, 18, 509.

Rumke, P. (1954) The presence of sperm antibodies in the serum of two patients with oligospermia. Vox Sanguinis, 4, 135.

Rümke, P. (1968) Sperm-agglutinating autoantibodies in relation to male infertility. Proc.Roy.Soc.Med., 61, 275.

Rümke, P. (1969) Clinical aspects of autoimmunity to spermatozoa in men. In Immunology & Reproduction Ed. R.G. Edwards. International Planned Parenthood Federation.

Rümke, P. & Hellinga, G. (1959) Autoantibodies against spermatozoa in sterile men. Am.J.Clin.Path., 32, 357.

Sainte-Marie, G. (1962) A paraffin embedding technique for studies employing immunofluorescence. J.Histochem.Cytochem., 10, 250.

Scheidegger, J.J., (1955) Une micro-méthode de l'immuno-électrophorèse Intern.Arch.Allerg.Appl.Immunol., 7, 103.

Schoysman, R. (1968) Treatment of male infertility due to auto-agglutination of spermatozoa. 6th World Congress of Fertility and Sterility, Tel Aviv, 112.

Schulman, S. (1969) Antigenic analysis of the male tract. In Immunology and Reproduction, p.111. Ed.R.G.Edwards. International Planned Parenthood Federation.

Schumacher, G.F.B. (1968) Protein analysis of secretions of the female genital tract. Lying in: The Journal of Reproductive Medicine, 1, 61.

Schwimmer, W.B., Ustay, K.A. & Behrman, S.J. (1967) Spermagglutinating antibodies and decreased fertility in prostitutes. Obstet.Gynec., 30, 192.

Searcy, R.L., Craig, R.G. & Bergquist, L.M. (1964) Immunochemical properties of normal and pathologic seminal plasma. Fert.Steril., 15, 1.

Segal, S., Tyler, E.T., Rao, S.S., Rumke, P. & Nakabayashi, N. (1961) Immunological factors in infertility. In 'Sterility'. Ed. E.T. Tyler, McGraw-Hill, New York.

Setchell, B.P. (1967) The blood-testicular fluid barrier in sheep. J.Physiol., Lond., 189, 63P.

Setchell, B.P. (1968) The effect of age, hypophysectomy and cryptorchidism on fluid secretion by the rat testis. Australian J.Exptl.Biol.Med.Sci., 46, 35P.

Setchell, B.P., Dawson, R.M.C. & White, R.W. (1968) The high concentration of free myo-inositol in rete testis fluid from rams. J.Reprod.Fert., 17, 219.

Setchell, B.P. & Linzell, J.L. (1968) Effect of some drugs and hormones on the secretion of fluid by the testis of the ram. J.Reprod.Fert., 16, 320.

Setchell, B.P., Voglmayr, J.K. & Waites, G.M.H.(1969) A blood-testis barrier restricting passage from blood into rete testis fluid but not into lymph. J.Physiol., Lond., 200,73.

Shahani, S. & Southam, A.L. (1962) Immunofluorescent study of the ABO blood group antigens in human spermatozoa. Am.J.Obstet. Gynec., 84, 660.

Shands, J.W. Jnr. (1967) In Modern Trends in Immunology 2. Ed. Cruikshank, R. & Weir, D.M. Butterworths.

Shetye, T.A. & Rao, S.S. (1968) Aspermatogenesis induced by epididymal extract and Freund's adjuvant in mice. Ind.J.Exp. Biol., 6, 123.

Shivers, C.A., Metz, C.B. & Lutwak-Mann, C. (1964) Some properties of pig follicular fluid. J.Reprod.Fert., 8, 115.

Simmons, R.L. & Russell, P.S. (1966) The histocompatibility antigens of fertilized mouse eggs and trophoblast. Ann.N.Y. Acad.Sci., 129, 35.

Smith, J.W., Barnett, J.A., May, R.P. & Sandford, J.P. (1967) Comparison of the opsonic activity of gamma G and gamma M anti Proteus globulins. J.Immunol., 98, 336.

Sokolovskaya, I.I. (1967) Experimental studies on reproduction by immunological methods. Int.Symp.Immun.Spermatozoa and Fertilization, Varna, p.63. Bulgarian Academy of Sciences.

Sokolovskaya, I.I. & Reshetnikova, N.M.(1968) Immune factors in fertilization and prenatal development. Animal Husbandry, 6, 65.

Sokolovskaya, I.I. & Reshetnikova, N.M.(1969) Investigations of immunobiological factors in fertilization and early development. In Immunology and Reproduction, p. 192. Ed.R.G. Edwards. International Planned Parenthood Federation.

Spar, I.L., Bale, W.F., Wolfe, D.E. & Goodland, R.L.(1956) Specificity of labelled kidney eluates. J.Immunol., 76, 119.

Spitznagel, J.K.(1966) Normal serum cytotoxicity for $P^{32}$-labelled smooth Enterobacteriaceae III Isolation of a $\gamma$-G normal antibody and characterization of other serum factors causing $P^{32}$ loss. J.Bact., 91, 401.

Spooner, R.L.(1964) Cytolytic activity of the serum of normal male guinea-pigs against their own testicular cells. Nature, 202, 915.

Steinberger, A. & Steinberger, E.(1967) Factors affecting spermatogenesis in organ cultures of mammalian testes. J.Reprod.Fert. Supplement No. 2, p.117.

Steinberger, E. & Duckett, G.E.(1967) Hormonal control of spermatogenesis. J.Reprod.Fert.Supplement No. 2, p.75.

Stern, L. & Peyrot, R. (1927) Le Functionnement de la barriere hematoencephalique aux divers stades de developpement chez divers espes animales. C.R.Soc.Biol., (Paris), 96, 1124.

Stevens, K.R., Hafs, M.D. & Kirton, K.T.(1964) Immunochemical and electrophoretic properties of oestrous rabbit uterine fluid proteins obtained by uterine ligation. J.Reprod.Fert., 8, 319.

Stone, S.M., Lerner, E.M. & Goode, J.M. (1968) Adoptive transfer of autoimmune aspermatogenesis in inbred guinea-pigs. Fed.Proc., 27, 363.

Störiko, K. (1968) Normal values for 23 different human plasma proteins determined by single radial immunodiffusion. Blut, 16, 200.

Straus, E.K. (1965) Sperm immobilization in the human vagina by induced mucoantibody. Fert.Steril., 16, 346.

Suvanto, O. & Kormano, M. (1968) Spontaneous contractility of the rat seminiferous tubule in relation to the stage of the cycle of the seminiferous epithelium. Scand.J.Clin.Lab. Invest., 21, Suppl. 101, 66.

Symonds, D.B.A. (1967) Reaction of spermatozoa with uterine serum globulin determined by immunofluorescence. J.Reprod.Fert., 14, 163.

Taylor-Robinson, D. & Manchee, R.J. (1967) Spermadsorption and spermagglutination by mycoplasmas. Nature, Lond., 215, 484.

Terry, W.B. (1965) Skin sensitizing activity related to polypeptide chain characteristics of human igG. Immunol., 95, 1041.

Tomasi, T.B. & Bienenstock, J. (1968) Secretory immunoglobulins. Advances in Immunol., 9, 2.

Tribulev, G.P., Podoplelov, I.I., Popivanov, R., Nakov, L.S., Zhivkov, S.M. & Vulchanov, V.H., (1967) Similarities and differences in the antigenic properties of spermatozoa and continuously cultivated cells in man. Int.Symp.Immun. Spermatozoa and Fertilization, Varna. Bulgarian Academy of Sciences.

Tyler, A. & Bishop, D.W. (1963) Immunological phenomena. In Mechanisms concerned with conception, p.397. Ed.C.G.Hartman. Pergamon Press.

Tyler, A., Tyler, E.T. & Denny, P.C. (1967) Concepts and experiments in immunoreproduction. Fert.Steril., 18, 153.

Vaughn, R.B. & Boyden, S.V. (1964) Interactions of macrophages and erythrocytes. Immunol., 7, 118.

Voglmayr, J.K., Scott, T.W., Setchell, B.P. & Waites, G.M.H. (1967) Metabolism of testicular spermatozoa and characteristics of testicular fluid collected from conscious rams. J. Reprod. Fert., 14, 87.

Voisin, G.A., Delaunay, A. & Barber, M.(1951) Lesions testiculaires provoquées chez le cobaye par injection d'extrait de testicule homologue. C.r.hebd.Séanc.Acad.Sci., Paris, 232, 1264.

Voisin, G.A. & Toullet, F. (1968) Étude sur l'orchite aspermatogenetique autoimmune et les autoantigenes de spermatozoides chez le cobaye. Ann.Inst.Pasteur, 114, 727.

Voisin, G.A. & Toullet, F. (1969) Relation between hypersensitivity responses to autoantigens and tissue damage in the male reproductive tract. In Immunology and Reproduction, p. 93. Ed. R.G. Edwards. International Planned Parenthood Federation.

Vojtiskova, M. (1969) H-$2^d$ antigens on mouse spermatozoa. Nature, 222, 1293.

Vojtiskova, M., Chutna, J., Rychlikova, M. & Pokorna, Z., (1962). On the possible role of immunological tolerance in the prevention of autoimmune aspermatogenesis. Folia Biol.(Praha), 8, 207.

Vulchanov, V.H.(1969) Testicular damage and autoantibody formation in guinea-pigs immunized with homologous seminal vesicular fluid. In Immunology and Reproduction, p.136. Ed.R.G. Edwards. International Planned Parenthood Federation.

Wadsworth, C. & Hanson, L. (1960) Comparative analysis of immune electrophoretic precipitates employing a modified immune electrophoretic technique. Int.Arch.Allerg.Appl.Immunol., 17, 165.

Waites, G.M.H. & Setchell, B.P. (1966) Changes in blood flow and vascular permeability of the testis, epididymis and accessory reproductive organs of the rat after administration of cadmium chloride. J.Endocr., 34, 329.

Waksman, B.H. (1959) A histologic study of the auto-allergic testis lesion in the guinea-pig. J.Exp.Med., 109, 311.

Waksman, B.H. (1960) The distribution of experimental auto-allergic lesions. Its relation to the distribution of small veins. Am.J.Path., 37, 673.

Walsh, L.S.N. (1925) Natural auto and homoio spermotoxins in guinea-pig serum. J.Immunol., 10, 803.

Weigle, W.O., High, G.J. & Nakamura, R.M. (1969) The role of mycobacteria and the effect of proteolytic degradation of thyroglobulin on the production of autoimmune thyroiditis. J.Exp.Med., 130, 243.

Weil, A.J. & Finkler, A.E. (1959) Isoantigenicity of rabbit semen. Proc.Soc.Exp.Biol.Med., 102, 624.

Weil, A.J. & Roberts, C.O. (1965) Fertility of female rabbits after isoimmunization with seminal plasma. Fert.Steril., 16, 355.

Weir, D.M., Pinckard, R.N., Elson, C.J. & Suckling, D.E. (1966) Naturally occurring antitissue antibodies in rat sera. Clin.Exp.Immunol., 1, 433.

Wiederhielm, C.A. (1966) Transcapillary and interstitial transport phenomena in the mesentery. Fed.Proc., 25, 1789.

Wilson, L. (1954) Sperm agglutinins in human semen and blood. Proc.Soc.Exp.Biol.Med., 85, 652.

Wilson, L. (1956) Sperm agglutination due to autoantibodies. A new cause of sterility. Fert.Steril., 7, 262.

Yamanaka, M.S. & Soderwall, A.L. (1960) Transport of spermatozoa through the female genital tract of hamsters. Fert.Steril., 11, 470.

Yanagamachi, R. (1969) In vitro capacitation of hamster spermatozoa by follicular fluid. J.Reprod.Fert., 18, 275.

Yanagimachi, R. & Chang, M.C. (1962) Infiltration of leucocytes into the uterine lumen of the golden hamster during the oestrous cycle and following mating. J.Reprod.Fert., 5, 389.

Yanagimachi, R. & Chang, M.C. (1963) Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. J.Reprod.Fert., 6, 413.

Yatvin, M.B. & Leathem, J.H. (1964) Origin of ovarian cyst fluid: studies on experimentally induced cysts in the rat. Endocrinology, 75, 733.

Yokoyama, M. & McCoy, J.E., Jr., (1968) Mild haemolytic disease of the newborn due to anti-Xga antibody. Am.J.Obstet.Gynec., 101, 859.

Yount, W.J., Dorner, M.M., Kunkel, H.G. & Kabat, A. (1968) Studies on human antibodies. VI. Selective variations in subgroup composition and genetic markers. J.Exp.Med., 12, 633.

Zachariae, F. & Jensen, C.E. (1958) Studies on the mechanism of ovulation. Histochemical and physicochemical investigations on genuine follicular fluids. Acta Endocr., 27, 343.